Apple Stem Grooving Virus (ASGV) and Apple Stem Pitting Virus (ASPV): Detection and isolate characterization in South African pome fruit

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Summary

Apple stem grooving virus (ASGV) and Apple stem pitting virus (ASPV) are known to infect pome fruit in all pome fruit producing regions of the world. In this study, a comparison between double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) for ASGV detection in pome fruit was performed. A total of 15 ASGV positive orchard leaf samples were detected using RT-PCR whilst DAS-ELISA could only detect 13. RT-PCR was found to be at least 126 fold more sensitive than DAS-ELISA.

In an assessment of the genetic variation of ASGV in South Africa, the coat protein (CP) gene of isolates was sequenced, aligned and phylogenetically analysed with ASGV CP gene sequences from GenBank. Parsimony analysis identified groups of isolates, but could not resolve the relationships between them. In order to obtain better resolution, whole genome sequences of international ASGV and *Citrus tatter leaf virus* (CTLV) isolates were aligned with ASGV and CTLV CP gene sequences and phylogenetically analysed with parsimony. South African ASGV isolates grouped into three clades and showed multiple origins and no geographical trend.

In an assessment of the genetic variation of ASPV in South Africa, the CP gene sequences of infected samples were aligned with international CP gene sequences obtained from GenBank and phylogenetically analysed using parsimony. Results from the analysis using parsimony revealed low CI and RI values indicating homoplasy in the CP gene data. To address the homoplasy, two additional analyses were performed in which the gene sequences were converted to amino acid sequences and in which the third position of the codon was excluded from the alignment. Both of these approaches resulted in a reduction in homoplasy. In an attempt to further increase the resolution of the phylogeny, the phylogenetic analysis was repeated using maximum likelihood. In the first codon unpartitioned analysis a tree with low support was retrieved followed by, as with the parsimony analysis, an analysis performed on the data translated to amino acid sequences, which showed better resolution and higher clade support. The tree with the highest resolution and clade support was retrieved by codon partitioning into first, second and third positions. South African ASGV isolates grouped into five clades and showed multiple origins and no geographical trend.

This study is the first in which ASGV and ASPV have been detected using RT-PCR in South Africa. Dual infections of ASGV and ASPV were recorded in 24.7% of samples analysed.

This is the first report of South African pear trees exhibiting symptoms of pear stony pit and fruit deformation associated with ASPV infection.

Opsomming

Apple stem grooving virus (ASGV) en Apple stem pitting virus (ASPV) kom wêreldwyd voor waar kernvrugte geproduseer word. In hierdie studie is ‘n vergelyking tussen die opsporingsgrense van “double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)” en “reverse transcriptase polymerase chain reaction (RT-PCR)” vir ASGV bepaal. RT-PCR kon ASGV opspoor in 15 ASGV geïnfekteerde kernvrug isolate terwyl die DAS-ELISA slegs ASGV kon opspoor in 13 van die isolate. Daar is gevind dat RT-PCR met ‘n minimum van 126 keer meer sensitief is as DAS-ELISA.

In ‘n ondersoek na die genetiese variasie van ASGV in Suid Afrika is die nukleotiedvolgorde van die mantelproteïngeen van isolate bepaal, in lyn geplaas- en filogeneties geanaliseer met volgordes wat van GenBank verkry is. ‘n Parsimony analise het isolaat groepe geïdentifiseer, maar kon nie die verhouding tussen hulle oplos nie. In ’n poging om beter resolusie te verkry is ASGV en “*Citrus tatter leaf virus* (CTLV)” heel genoom volgordes in lyn geplaas met die mantelproteïn volgordes en filogeneties geanaliseer met parsimony. Suid-Afrikaanse isolate groepeer in drie klades van veelvuldige oorspronge wat dus geen geografiese tendens aandui nie.

In ’n ondersoek na die genetiese variasie van ASPV in Suid-Afrika is die nukleotied volgorde van die mantelproteïngeen van isolate bepaal, in lyn geplaas- en filogeneties geanaliseer met ASPV volgordes wat van GenBank verkry is. ‘n Parsimony analise het isolaat groepe geïdentifiseer maar kon nie die verhouding tussen hulle oplos nie. Lae CI en RI waardes het aangedui dat daar homoplasie in die data van die mantelproteïngeen is. Die resultate van twee addisionele analises, waarin geenvolgordes onderskeidelik omgeskakel is na aminosuurvolgordes en die derde posisie van die kodon uitgesluit is in die volgorde, dui ‘n afname in homoplasie aan. In ’n poging om die resolusie van die filogenie te verbeter is die filogenetiese analise herhaal deur “maximum likelihood” te gebruik. In die eerste analise, waar die kodon nie verdeel is nie, is ’n boom met lae ondersteuning opgespoor. Dit is opgevolg met, net soos by parsimony, ’n analise van data wat omgeskakel is na aminosuurvolgorde. ’n Boom met hoër resolusie en waarvan die klades beter ondersteun is, is verkry. Die boom met die hoogste resolusie en klade ondersteuning is opgespoor deur die verdeling van die kodon in eerste, tweede en derde posisies. Suid-Afrikaanse isolate groepeer in vyf klades van veelvuldige oorspronge wat dus geen geografiese tendens aandui nie.

Hierdie is die eerste studie waarin die teenwoordigheid ASGV en ASPV met behulp van RT-PCR in Suid-Afrika bepaal is. Dubbel-infeksie van ASGV en ASPV is verkry in 24.7% van

monsters wat in die studie geanaliseer is. Hierdie is die eerste verslag op rekord van Suid-Afrikaanse pere wat simptome van peerverpitting en vrugvervorming toon wat geassosieer is met ASPV infeksie.

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Abbreviations

ACLSV	Apple chlorotic leafspot virus
ALV	Apricot latent virus
ApMV	Apple mosaic virus
ASGV	Apple stem grooving virus
ASPV	Apple stem pitting virus
bp	base pair
BLAST	basic local alignment search tool
cDNA	complementary DNA
CI	consistency index
CP	coat protein
CTLV	Citrus tatter leaf virus
DDBJ	DNA DataBank of Japan
DNA	deoxyribonucleic acid
ds-cDNA	double stranded cDNA
dsDNA	double stranded DNA
dsDNA-RT	double stranded DNA-reverse trancriptase
dsRNA	double strandeded RNA
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPS	encapsulated postscript format
IC-RT-PCR	immunocapture reverse transcriptase polymerase chain reaction
kb	kilobases
kDa	kiloDalton
Mabs	monoclonal antibodies
MP	movement protein
M_r	molecular weight
mRNA	messenger RNA
nm	nanometer
nt	nucleotide

ORF	open reading frame
PASV	Peach asteroid spot virus
PAUP	Phylogenetic Analysis Using Parsimony
PBNLS	Pear black necrotic leaf spot
PCR	polymerase chain reaction
PVT	Potato virus T
PVYV	Pear vein yellow virus
RdRp	RNA-dependent RNA polymerase
RI	retention index
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SAPO	South African plant improvement organization
SSCP	single strand conformation polymorphism
ssDNA	single stranded DNA
sgRNAs	subgenomic RNAs
ssRNA	single stranded RNA
ssRNA+	positive sense single stranded RNA
ssRNA-	negative sense single stranded RNA
ssRNA-RT	single stranded RNA-reverse trancriptase
TBR	tree bisection and reconstruction
TGBp	triple gene block protein

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Chapter 1. Introduction

In South Africa, the tradition of growing fruit at the Cape of Good Hope dates back to the late 1650s when the first trees were planted to supply fruit to passing ships. For the next two centuries, fruit production flourished and fruit was sold in towns that grew around the discovery of diamonds and gold. The domestic market was however limited. In the late 1800s the first attempt was made to export fruit to England. In order to produce healthy fruit for exportation, American nurseryman Harry Pickstone, with financial backing from Cecil John Rhodes, imported 50 000 fruit trees from California which were established in the Western Cape region (Hurndall, 2005).

In South Africa, deciduous fruit is produced on approximately 76 000 hectares. The replacement value of the industry is estimated to be R9.3 billion. In 2002, the gross export earnings were approximately R8.1 billion, of which table grapes, followed by apples and pears were the largest contributors. Pome fruit in South Africa are cultivated and/or produced in the Eastern-, Northern-, and Western Cape, Free State, Gauteng, KwaZulu Natal and Mpumalanga (Hurndall, 2005).

The local market has always been important to the South African fruit industry. With urbanization rapidly increasing and a growing population, there is a continuous, ever-increasing demand for fresh fruit. In addition to the municipal and local markets, retail chain stores, greengrocers, cafes, thousands of independent stallholders, hawkers and vendors distribute fruit to consumers throughout the country. Today, fruit is available throughout the year in almost every populated region of South Africa. There are many brand names for deciduous fruit and a wide variety of cultivars, but they all have one feature in common: a minimum of grading and quality standards (Hurndall, 2005).

Viral infection of fruit trees has a direct effect on fruit yield and therefore on the income of fruit production. Planting healthy fruit trees for the export fruit market is of significant economic importance to South Africa. Viruses tend to shorten the lifespan of trees and cause a decrease in fruit yield which in turn contributes to a substantial loss of income (Devignes and Boyé, 1989). A study done by Cembali *et al.*(2003) showed yield losses of 12-30% in ASGV infected Golden Delicious apple orchards. According to the annual Hortgro tree census of 2013, fruit trees in South Africa cover 77 805 hectares of which 33 481 hectares (ha) are pome fruit. In turn, it provides labor to approximately 106000 laborers with approximately 420000 dependants of which approximately 43000 labourers and their approximately 170 000 dependants are allocated to pome fruit production. During 2013 a total of 358 457 tons of apples and 177 848 tons of pears were exported (Kotze, 2013).

Fruit trees are susceptible to diseases at every step in their production and distribution chain, with economic losses occurring at the nursery, in producers' fields, during storage, after harvest and in the marketplace. With higher standards of plant material come an increasing need to control the sanitary conditions of fruit trees due to the large number of diseases caused by viruses, phytoplasmas and other harmful organisms. Plant certification and the continuous process of introducing new cultivars to the fruit industry are key factors for increasing the market pressure of competitiveness between different fruit companies. In South Africa today, the deciduous fruit industry operates under the South African Deciduous Fruit Plant Certification Scheme as described by Law 51 of 1976 to produce certified plant material that yields healthy fruit of high quality (http://www.plantsa.co.za/spv.php).

The distribution of plant material certified to be free of specific diseases is the main aim of plant improvement. The South African Deciduous Fruit Plant Certification Scheme currently prescribes that pome fruit should be free of ASGV and ASPV. Currently, the scheme prescribes that new imported pome fruit varieties are tested with the ELISA technique for ASGV pending candidate registered status. However, ELISA testing for ASPV is not compulsory. New imported varieties subsequently have to undergo the process of biological indexing for ASGV and ASPV to achieve registered clone status. Biological indexing, which is conducted over a period of two growing seasons , is extremely time consuming in a fast-growing industry where time is of outmost importance. There is an urgent need in the industry to speed up these testing procedures and also to increase the sensitivity of the methods used for testing. For these reasons, the first objective of this study was to detect ASGV with the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) method as currently prescribed and to compare the sensitivity of the method to the more sensitive reverse transcriptase polymerase chain reaction (RT-PCR) methods. The second objective was to identify ASPV using RT-PCR. The third objective was to determine the genetic variation of the ASGV and ASPV isolates present in South Africa in comparison to isolates occurring elsewhere in the world.

In order to address these objectives, the thesis is structured accordingly. In Chapter 2, an overview is given of the influence that the pome fruit viruses ASGV and ASPV has on fruit trees, the quality of plant propagation material as well as the fruit yield. It also focuses on the different testing methods and technologies currently available for the detection of these viruses worldwide. Furthermore an overview is given of phylogenetic analyses to determine genetic variation of the viruses that occur in South Africa. In Chapter 3, a comparison of the DAS-ELISA method with the RT-PCR method for the detection of ASGV is presented. In Chapter 4, results are presented for determining the genetic variation of ASGV isolates in

South Africa by means of ASGV coat protein (CP) gene sequencing and phylogenetic analysis as well as a phylogenetic analysis of South African ASGV CP gene sequences and ASGV CP gene- and whole genome sequences from other areas of the world. In Chapter 5, results a presented for RT-PCR used for the detection of ASPV isolates. In order to detect the genetic variation of the ASPV isolates that occur in South Africa, a phylogenetic analysis was performed on the CP gene sequences of South African ASPV isolates and CP gene sequences from other parts of the world. The conclusion and future perspectives are presented in Chapter 6.

Chapter 2. Literature review of Apple stem grooving virus and Apple stem pitting virus on pome fruit trees

2.1. Introduction

The discovery of the first virus dates back to the late 1800s with the discovery of what is known today as *Tobacco mosaic virus* (TMV). In 1892, Russian scientist Dmitri Ivanowski reported that extracts from tobacco leaves with mosaic symptoms were still infectious after filtration through a Chamberland filter-candle. Ivanovski however concluded that the agent was either a toxin or bacterial in nature. In 1898, Dutch scientist Martinus van Beijerinck did similar experiments and was the first to call what we know today as a plant virus, incitant of the tobacco mosaic. He concluded that the incitant was able to migrate in an agar gel, therefore being an infectious soluble agent and definitely not bacteria. The discovery of TMV is seen as a milestone in the discovery of viruses (Lecoq, 2001).

Viruses, such as TMV, are small infectious intracellular pathogenic particles that infect other living organisms. These obligate intracellular parasites, meaning they require a living host in order to grow and multiply, infect all types of life forms, including animals, plants, bacteria and archaea. Since the cell membrane of a plant cell is surrounded by a rigid cell wall, plant viruses require a wound for their initial entry into a plant cell. Wounds can occur naturally, such as in branching of lateral roots, hail, rain and/or wind damage. They may also be the result of agronomic or horticultural practices, or other mechanical means; fungal, nematode or parasitic plant infections, or by insects. Organisms that transmit pathogens such as viruses are called vectors. Viruses can also be transmitted mechanically e.g. by using infected pruning equipment (Ellis *et al*, 2008).

Once inside the plant cell, the virus particle sheds its protein coat and the nucleic acid after which it directs the production of multiple copies of itself. Plant viruses move systemically throughout infected plants via the phloem (Ellis *et al*., 2008). Virus incidence greatly differs according to the location of plantings. Trees from healthy mother-tree material have a higher risk of viral infection when planted near infected commercial orchards than those planted in isolated areas (Varveri and Bem, 1995). Although most viruses show visual fruit or leaf symptoms on infected plants that can be identified during orchard monitoring, some viruses, called latent viruses, show no symptoms on most commercial fruit varieties, although symptoms may occur in some susceptible varieties such as Virginia crab apple being a susceptible host for ASGV expression. Seasonal testing, the use of sanitized pruning and indexing equipment, as well as orchard monitoring and eradication of virus infected trees are

important to lower the chances of cross contamination by mechanical and/or vector transmission.

In recent years, a number of economically important viruses on fruit trees have been characterized and their genome sequences are available on public databases such as GenBank, EMBL and DDBJ. This study presents an overview of the influence of Apple stem grooving virus (genus *Capillovirus*) and Apple stem pitting virus (genus *Foveavirus*) on pome fruit trees. It includes a description of the structure of each virus. Both ASGV and ASPV are latent viruses meaning they do not show any symptoms on most of the commercially produced varieties. It was therefore also important to include the host range, distribution and symptom expression (shown only in susceptible varieties) of both viruses. Furthermore this overview also provides the reader with a thorough description of three testing methods commonly used for the detection of these viruses in certification schemes around the world. They are the biological indexing-, DAS-ELISA- and RT-PCR methods.

2.2. Virus structure

Virus particles (known as virions) consist of two or three parts: i) the inner core of nucleic acid, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), that carry genetic information; ii) an outer sheath or protein coat (referred to as the capsid) that protects these genes; and in some cases iii) an envelope of lipids that surrounds the protein coat when they are outside a cell. Viruses can be divided into different classification groups according on their unique characteristics (Figure 2.1) (Collier *et al*., 1998):

- **DNA viruses** – A DNA virus is a virus that has DNA as its genetic material and replicates using a DNA-dependent DNA polymerase. DNA viruses group into double-strand DNA (dsDNA)- and single-strand DNA (ssDNA) viruses.

- **RNA viruses** – A RNA virus is a virus that has RNA (ribonucleic acid) as its genetic material. RNA viruses can be grouped into *double-strand RNA* (dsRNA)- and single-strand RNA (ssRNA) viruses. Single-stranded RNA viruses contain either a positive RNA strand (ssRNA+) or a negative RNA strand (ssRNA-). After entering the host cell, the virion uncoats in the cytoplasm, releasing the ssRNA. Since the positive RNA strand is already in the form of messenger RNA, it can be translated immediately into capsomere proteins used to assemble new viral capsids. Replication of the positive RNA strand is accomplished in two steps. Firstly, the RNA polymerase creates a template strand through complementary base pairing which in turn is used to make many complementary positive RNA strands. This newly made

positive strands combine with the capsids to complete the construction of new positive, single-stranded RNA virions. Unlike ssRNA(+), the ssRNA(-) is not in the form of messenger RNA. Therefore RNA polymerase must first make template strands through complementary base pairing to form messenger RNA which can be translated into capsomere proteins used to assemble new viral capsids. The template strands are also used to produce new negative RNA strands. The newly made negative RNA strands combine with the capsids to complete the construction of new, negative stranded RNA virions. Both ASGV and ASPV are ssRNA(+) viruses).

- **Retro-transcribing viruses** – Retro-transcribing viruses include single strand RNA-reverse trancriptase (ssRNA-RT)- and double strand DNA-reverse trancriptase (dsDNA-RT) viruses. Viruses from this group replicate inside cells they have invaded through an RNA intermediate called reverse transcriptase.

The protein shells of plant viruses (capsids) are assembled in accord with one of two fundamental types of symmetry. The first shape of virion is helical (roughly elongated) and come in two major variants: rigid rods (Figure 2.2A) and flexuous filaments (Figure 2.2B). In both these variants, the nucleic acid is highly ordered, assuming the same helical conformation as the proteinaceous capsid. The second shape of virus particle is icosahedral (roughly spherical; Figure 2.2C). The variants of this basic shape include bacilloform virions (Figure 2.2D) and twin virions composed of two joined incomplete icosahedra (Figure 2.2E). In the icosahedral virions the nucleic acid forms a partially ordered ball inside the proteinaceous capsid (Gergerich and Dolja, 2006). The average virus is about one one-hundredth the size of the average bacterium which is extremely small. Virus particles can only be seen with an electron microscope (Ellis *et al*., 2008)

The genus *Capillovirus* (member ASGV) and *Foveavirus* (member ASPV) both belong to the family Flexiviridae (Figure 2.1) (Faguet, 2002). In 2008, Barone *et al*. launched an investigation on the occurrence of Capillo- and Foveaviruses in ancient fruit tree cultivars in Campania, Southern Italy. Both ASGV and ASPV were detected in these cultivars providing information on the prevalence of Capillo- and Foveaviruses in old local cultivars in Italy. It also extends our knowledge on the ancient presence and diversity of these agents in fruit trees.

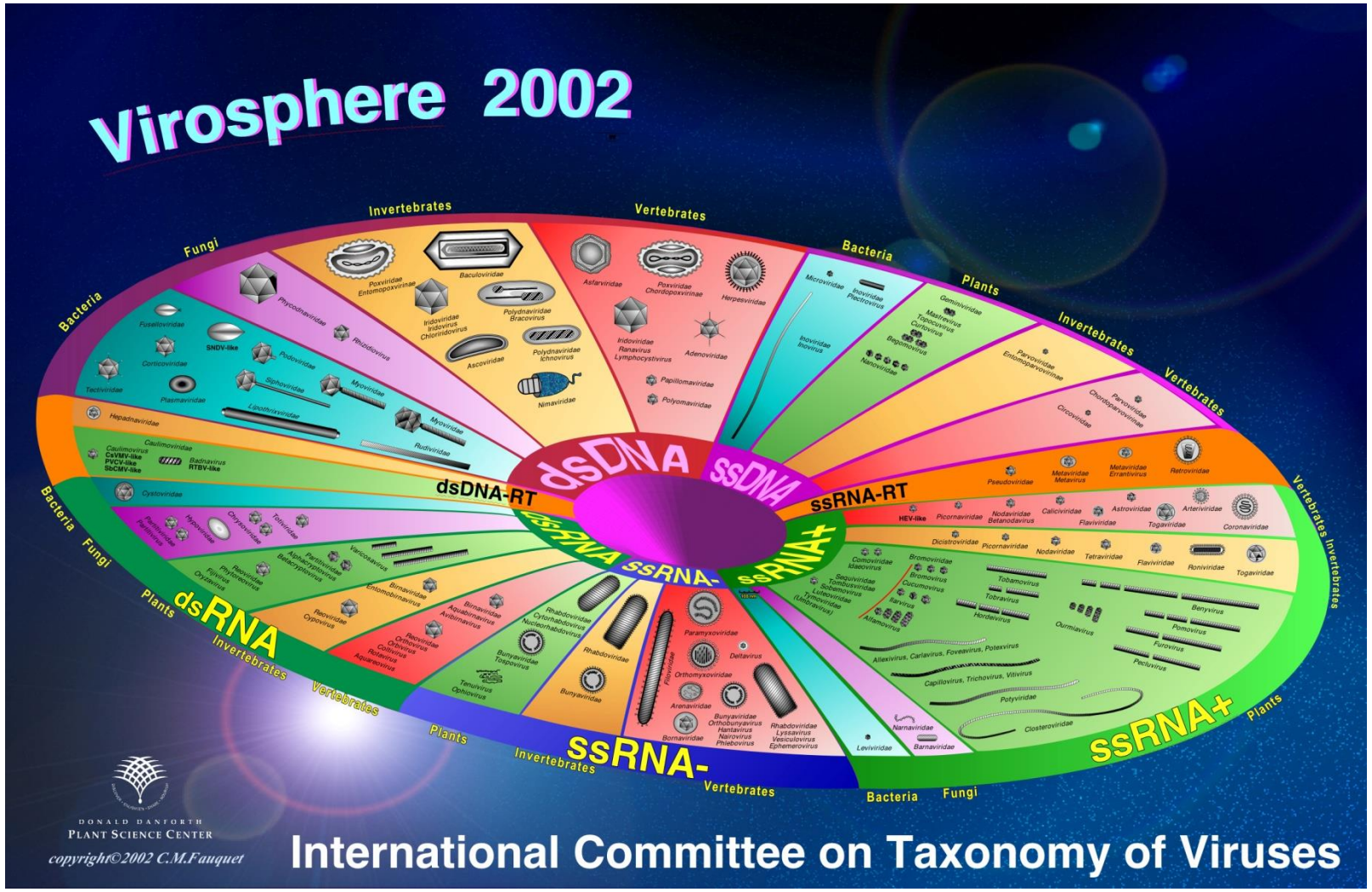

Figure 2.1. The different viruses divided into classification groups according to their unique characteristics. The arrow indicates the classification of the genus *Capillovirus* (member ASGV) and *Foveavirus* (member ASPV), both belonging to the family Flexiviridae. Both ASGV and ASPV consist of ssRNA(+) (Fauguet, 2002).

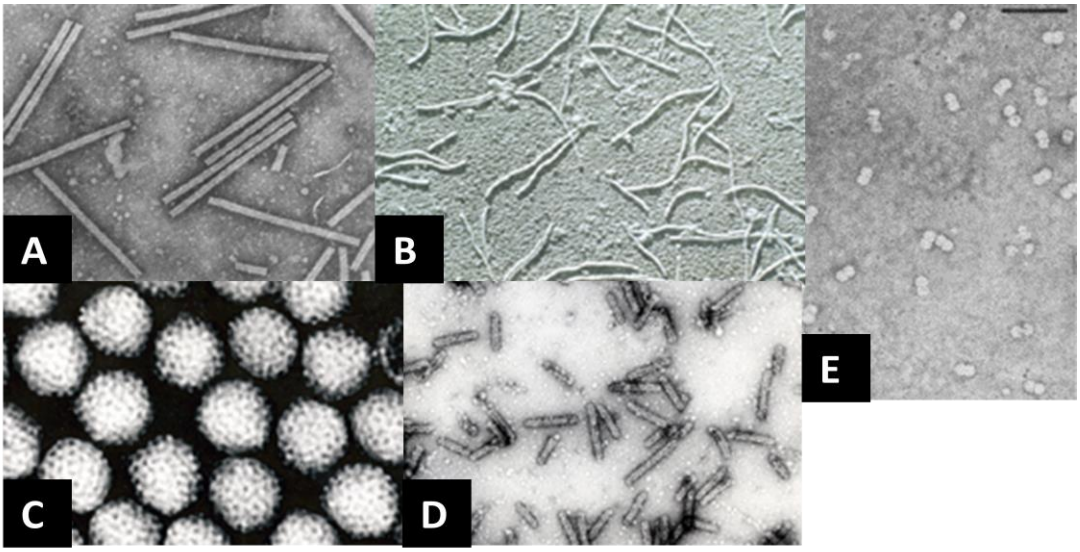

Figure 2.2: Electron microscopic images of the two fundamental types of virus particles: The helical and the icosahedral virions. Helical virions consist of rigid rods (A) of flexuous filaments (B). Icosahedral virions are roughly spherical (C). The variants of this basic shape include bacilloform virions (D) and twin virions composed of two joined incomplete icosahedra (E) (Gergerich and Dolja, 2006).

The family Flexiviridae was first described as a new virus family in 2004 and includes the existing genera *Allexivirus*, *Capillovirus*, *Carlavirus*, *Foveavirus*, *Potexvirus*, *Trichovirus*, *Vitivirus* and *Mandarivirus*. It was named after the feature that its members have flexuous virions and this was justified by phylogenetic analysis of the polymerase and CP sequences (Adams *et al*., 2004). Furthermore, members of the family Flexiveridae show similarities in virion morphology, have common features in genome type and organization, and there is a strong phylogenetic relationship between replicational- and structural proteins (Martelli *et al*., 2007).

2.2.1. Apple stem grooving virus (ASGV)

Apple stem grooving virus is a member of the *Capillovirus* genus. Plant viruses belonging to the genus *Capillovirus* are viruses with helically constructed filamentous particles of approximately 640 nm long and 12 nm in diameter. Virions have a positive sense, single-stranded RNA genome (6.5-7.5 kb in size) of which the 3' terminus is polyadenylated. The virion RNA is infectious and serves as both the genome and the viral messenger RNA (mRNA). For gene expression, RNA-dependent RNA polymerase (RdRp) is translated directly from the genomic RNA. The viral RNA is translated as a monocistronic mRNA to produce the RdRp. Replication occurs in that double stranded RNA (dsRNA) is synthesized from the genomic ssRNA(+). The dsRNA genome is then transcribed/replicated thereby providing viral mRNAs of new ssRNA(+) genomes. Internal subgenomic promoters are used

to transcribe subgenomic RNAs (sgRNAs). Transduction of these sgRNAs yields the CP and the movement protein (MP). MP allows cell-to-cell movement of new virus particles (Figure 2.3 A and B) (Viralzone, 2008).

Members of the *Capillovirus* genus encode two overlapping open reading frames (ORFs). ORF1 encodes a large polyprotein containing replication-associated protein plus a CP. ORF2 encodes a MP located within ORF1 in a different reading frame (Tatineni *et al*., 2009). The CP is the most abundantly expressed viral protein since large quantities are required for virion formation (Callaway *et al*., 2001). Within the Flexiviridae family, only members of the genus *Capillovirus* do not encode the CP as a separate ORF. Instead, it is located at the C-terminal region of a polyprotein encoded by ORF1. The organization of the CP sequence as part of the replicase ORF is therefore unusual in Capilloviruses (Martelli *et al*., 2007; Tatineni *et al*., 2009). How do Capilloviruses over produce enough CP from a polyprotein for virion formation? Although there is no significant evidence to date, one suggestion is that, as observed with members within the potyvirus group, surplus replication-related proteins accumulate as inclusion bodies in order to provide for virion formation (Dougherty and Carrington, 1988).

A: VIRION

B: GENOME

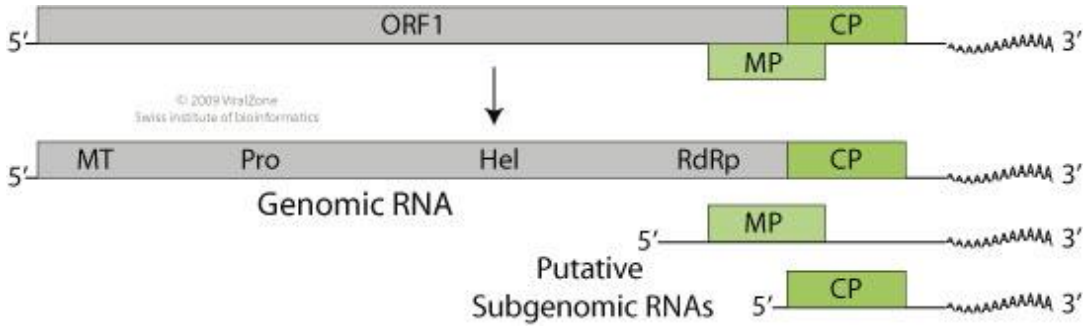

Figure 2.3: A. A presentation of the *Capillovirus* virion as a non-enveloped, flexuous and filamentous particle. It is 640 nm long and 12 nm in diameter. B: A presentation of the *Capillovirus* genome as a linear ssRNA(+) genome of 6.5-7.5 kb in size. The 3' terminus is polyadenylated (Viralzone, 2008).

Apple stem grooving virus has flexuous thread-like particles, approximately 600-700 x 12 nm (Figure 2.4) (Clover *et al*., 2003). ASGV contains a single RNA species with a relative

molecular weight (M_r) of 2.3 x 10^6. The CP gene contains 714 nucleotides, coding for a protein of 237 amino acids with a predicted M_r of 27 000. The single RNA species of ASGV is plus-sense and polyadenylated (3'-end) (De Sequeira and Lister, 1969; Yoshikawa and Takahashi, 1988; Nickel *et al*., 2001).

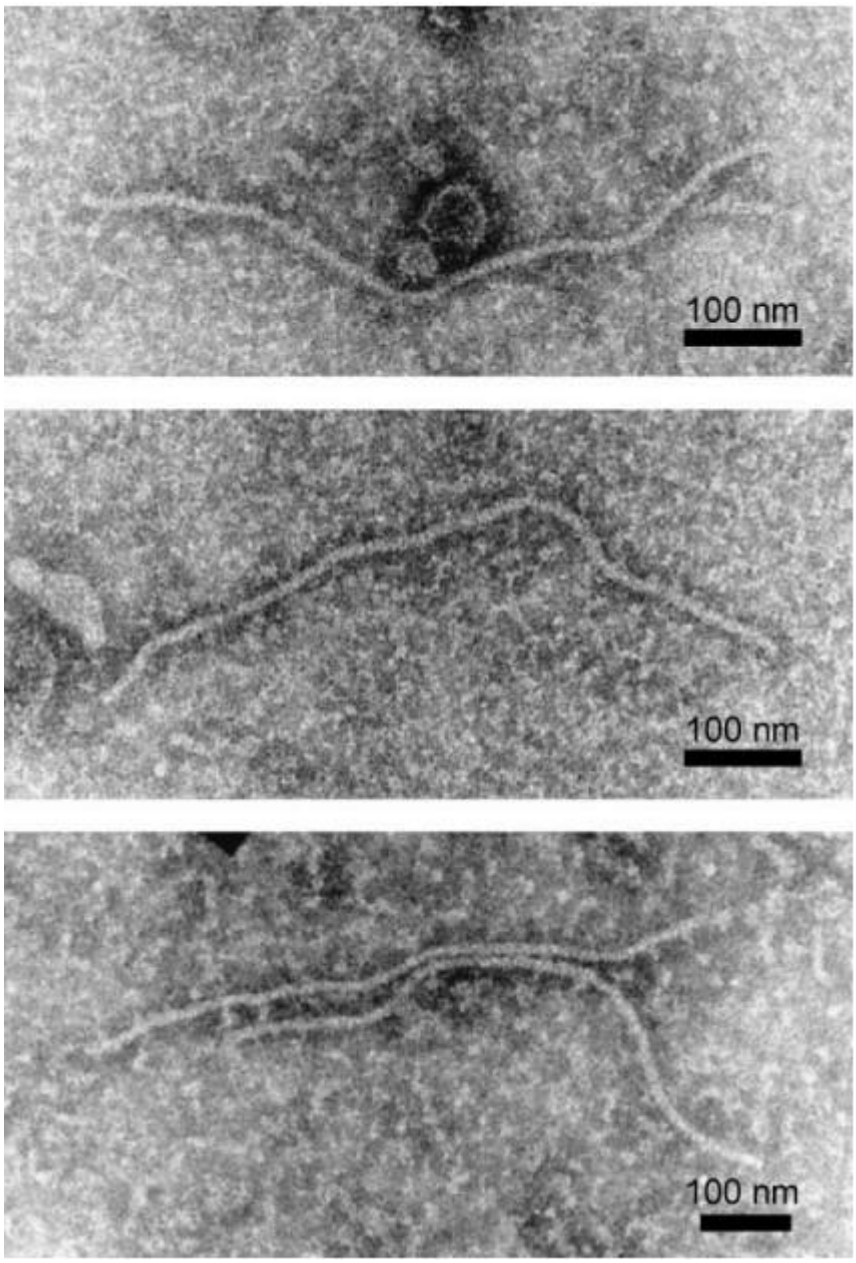

Figure 2.4: Electron micrograph of virus particles of ASGV in leaf sap showing its very flexuous, thread-like virus particles (Clover *et al*., 2003).

The genome of ASGV is 6495-6497 nucleotides in length excluding a 3'-terminal poly(A) tail, contains two overlapping ORFs and untranslated regions at the 5'-(35-36 nt) and 3'-(143 nt) of the genome. At the 5'-end of the ASGV genome is a cap that appears to be essential for infection. ORF1 begins at nucleotide position 37 and is terminated at base pair (bp) 6341,

encoding a 241 kDa protein. ORF2 is located in a different reading frame within ORF1. It begins at bp 4788 and can encode a 36 kDa protein similar to a putative movement protein. The 241 kDa protein of ORF1 contains two consensus sequences associated with the RdRp and the NTP-binding helicase. If the amino acid sequences in the vicinity of these conserved motifs are compared with those of other viruses, this shows the extensive similarities ASGV has with apple cholorotic leaf spot-, tymo-, carla-, and potex viruses. This indicates ASGV to be a member of the sindbis-like supergroup. The 36 kDa protein encoded by ORF2 contains the consensus sequence Gly-Asp-Ser-Gly found in the active site of several cellular and viral serine proteases (Yoshikawa *et al*., 1992). The CP gene variability among *Capillovirus* isolates from distinct regions is very low (Nickel *et al*., 2001). The CP of Indian ASGV isolate from the apple variety Starkrimson has 100% identity with the Brazilian isolate AF438409 (Negi *et al*., 2010).

The biochemical properties of ASGV are very closely related to those of *Potato virus T* (PVT, genus *Capillovirus*), although the genome organizations of these two viruses are quite different (Yoshikawa and Takahashi, 1988). ASGV also shows striking genome similarities with *Citrus tatter leaf virus* (CTLV, genus *Capillovirus*). In comparison with PVT, the sequence of the 3'-terminal 2956 nucleotides, (excluding the poly(A) tail) of the CTLV genome shows 86.1% similarity to that of the ASGV genome. Similarities of amino acid sequences encoded by ORF1 and ORF2 of CTLV with the corresponding regions of ASGV are 86.1% and 97.3% respectively. Very high amino acid identities of 95-100% have also been found between ASGV and CTLV, indicating CTLV to be a very close relative of ASGV within the genus *Capillovirus* (Yoshikawa *et al*., 1993; Negi *et al*., 2009).

The first report of a single silent nucleotide substitution in an ASGV isolate genome causing the attenuation of virus symptoms was published by Hirata *et al*. in 2003. The specific ASGV isolate, ASGV-RM21, whose *in vitro* transcript does not induce any symptoms characteristic of the original complementary DNA (cDNA) clone (ASGV wild type) in several plant hosts, has only a single, translationally silent nucleotide substitution, U to C, at nucleotide 4646 of the viral genome within ORF1. Northern- and western blot analysis showed that less of the ASGV-RM21 isolate accumulates in host plants than in the ASGV wild type. Using site-directed mutagenesis, two additional silent substitutions, U to A and U to G, were constructed at the same nucleotide (4646). Both additional substitutions showed induced attenuated symptoms. These results also implicate a novel determinant of disease symptom severity.

Magome *et al*. (1999) characterized the population of sequence variants within a single tree by applying a combination of an immunocapture RT-PCR (IC-RT-PCR) and a single-strand

conformation polymorphism (SSCP) of a asymmetric PCR product from ASGV infected apple, Japanese pear and/or European pear. The SSCP analysis results showed ASGV to exist as a mixture of sequence variants. The composition of sequence variants were different among leaf samples from different branches, indicating each sequence variant to be unevenly distributed within an individual tree. This study also showed some sequence variants to be dominant while others decreased, by changes in their decomposition, to undetectable levels within *Chenopodium quinoa* plants.

2.2.2 Apple stem pitting virus (ASPV)

Apple stem pitting virus is a member of the *Foveavirus* genus. The genus *Foveavirus* was first published as a new plant virus genus in 1998 (Martelli and Jelkmann, 1998). Plant viruses belonging to the genus *Foveavirus* are viruses with helically constructed filamentous particles of approximately 800 nm long (Figure 2.5 B). Virions have a positive sense, single-stranded, polyadenylated RNA genome (8.4-9.3 kb in size) and a single type of CP with a size of 28-44 kDa (Figure 2.5 A) (Viralzone, 2008). The virion RNA is infectious and serves as both the genome and the viral mRNA. For gene expression, RdPd is translated directly from the genomic RNA. Cytoplasmic replication takes place when the virus penetrates into the host cell. As soon as it becomes uncoated, viral genomic RNA is released into the cytoplasm. The viral RNA is translated as a monocistronic mRNA to produce the RdRp. Replication occurs in viral factories where a dsRNA is synthesized from the genomic ssRNA(+). The dsRNA genome is then transcribed/replicated thereby providing viral mRNAs of new ssRNA(+) genomes. Internal subgenomic promoters are used to transcribe sgRNAs. Translation of these sgRNAs yield the CP and movement protein (MP). Triple gene block proteins (TGBp) allow cell-to-cell and long distance movement of new virus particles (Figure 2.5A and B) (Viralzone, 2008b).

The genome consists of five ORF's with ORF 1 encoding for the replication-related proteins, ORF 2-4 for the putative movement proteins and ORF 5 for the CP. Virions do not contain lipids or carbohydrates and accumulate in the cytoplasm, where viral replication is likely to occur. The process of replication is based on direct expression of the 5'-proximal ORF and the expression of the downstream ORF's through sub-genomic RNA's. This replication process is similar to that of potexviruses. Apart from its similarities to the genus *Potexvirus*, the genome structure and organization of the genus *Foveavirus* also closely resembles that of genera *Carlavirus* and *Allexvirus* with the exception of ORF1 and the CP cistron (ASPV only) which are significantly larger in the genus *Foveavirus* (Martinelli and Jelkman, 1998).

Apple stem pitting virus is a flexuous, filamentous virus that is 12-15 nm wide and 800 nm long and is found in the cytoplasm of mesophyll cells. The virus is comprised of a single stranded positive-sense RNA with a M_r of 3.1 x 10^6 and a CP of M_r 48 000 (Koganezawa and Yanase, 1990). The complete CP gene consists of 1194 nucleotides and encodes a polypeptide of 394 amino acids (Liu and Niu, 2011). The complete genomes of ASPV of different isolates that have been sequenced vary between 9265 and 9310 nucleotides (Jelkman, 1994; Yoshikawa *et al*., 2001; Liu *et al*., 2012).

A: VIRION

B: GENOME

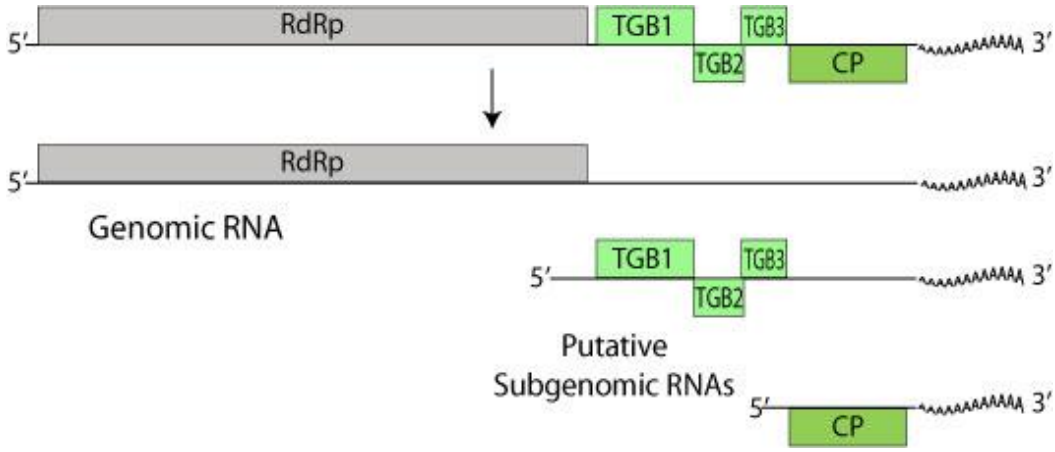

Figure 2.5: A. A presentation of the *Foveavirus* virion as a non-enveloped, flexuous and filamentous particle. It is at least 800 nm long and 12-15 nm in diameter. B. A presentation of the *Foveavirus* genome as a linear ssRNA(+) genome of 8.4-9.3 kb in size. The 3' terminus is polyadenylated and the 5'end is capped (Viralzone, 2008).

The genome of ASPV isolate PA66 (Jelkman, 1994) is 9306 nucleotides long and is composed of five open reading frames (ORFs) encoding putative proteins with a M_r-values of 247 000 (ORF1), 25 000 (ORF2), 13 000 (ORF3), 7 000 (ORF4), and 44 000 (ORF5), and has a poly(A) tail. In 2001, Yoshikawa *et al*. determined the complete nucleotide sequence of isolate IF38 and compared it to isolate PA66 sequenced by Jelkman (1994). The IF38 genome consists of 9293 nucleotides (nt), excluding the 3' poly (A) tail, 15 nucleotides fewer than that of the isolate PA66 genome. Analysis of the putative ORFs of isolate IF38 showed that the genome also contains five ORFs in the positive strand, encoding proteins with M_r-values of 247 000 (ORF1), 25 000 (ORF2), 13 000 (ORF3), 7 000 (ORF4), and

42 000 (ORF5). This study also showed consistency with the known genome heterogeneity of ASPV as reported earlier by Magome *et al*. (1997). Comparison of the nucleotide sequence of the IF38 genome and the PA66 genome showed a high variability, with 76% identity between the two isolates. The amino acid number of IF38 CP is 18 amino acids fewer than that of isolate PA66 as a result of deletions in the N-terminal region of the IF38 CP in ORF4.

Compared to potex, carla-, clostero-, and capilloviruses, the ASPV genome organization appears to be most closely related to that of potexviruses, but with a larger CP of M_r 44 000 (ORF5) (Jelkmann, 1994). The genome of *Apple latent virus* (ALV) contains nucleotide sequences related to that of ASPV with its 3' half being 77% identical to the corresponding region of ASPV (Nemchinov and Hadidi, 1998). Sequenced amplification products of various ASPV isolates revealed considerable sequence variation and deletions. This could be the reason for differences found in the sizes of PCR products of the CP gene (Schwartz and Jelkmann, 1998). Sequencing and hybridization analysis revealed a potentially high level of molecular variability among different isolates of the virus (Nemchinov *et al*., 1998).

2.3. Host range, distribution and symptom expression

Pome fruits are hosts of many viruses including Apple stem grooving virus (ASGV, genus *Capillovirus*) and Apple stem pitting virus (ASPV, genus *Foveavirus*) (James, 1998). Both viruses are distributed worldwide wherever pome fruit is cultivated. The genus *Foveavirus* was first published as a new plant virus genus of pome fruit in 1998 (Martelli and Jelkmann, 1998). There is no known vector for ASGV and ASPV and both viruses can be transmitted mechanically or by the use of infected material for grafting.

In 2004, it was reported that pear black necrotic leaf spot (PBNLS) disease could be caused by ASGV (Shim *et al*., 2004). In 2006, a further study by Shim *et al*. examined the pathogenicity of the ASGV Korean isolate ASGV-K to pear trees and other experimental hosts when carried by the fungal vector *Talaromyces flavus*. ASGV-harboring *T. flavus* induced mild PBNLS symptoms on virus-free pears. It may be that *T. flavus* is a fungal vector for ASGV.

Apart from the well-known pome fruit hosts apple and pear, ASGV and ASPV have also been reliably detected in quince (Mathioudakis *et al*., 2006; Negi *et al*., 2010). Most commercially grown apple and pear varieties, however, remain symptomless when infected by ASGV and ASPV. In susceptible apple, pear and quince varieties, ASGV and ASPV are associated with economically important diseases such as apple stem pitting, apple epinasty

and -decline, pear red mottle, pear stony pit, quince sooty ring spot and fruit deformities (Leone *et al*., 1998; Desvignes *et al*., 1999). Symptom expression on susceptible infected varieties includes chlorosis, leaf distortion and a non-specific gloss to the cultivar. However, these symptoms may vary in their intensity between different cultivars (Maxim *et al*., 2004).

Apple stem grooving virus is a stem grooving virus causing stem grooving, brown line and graft union abnormalities in Virginia crab apple. ASGV also has a negative influence on tree growth. Maxim *et al*. (2004) showed that the average tree height of artificially ASGV infected nursery trees was 23.4% lower and the average diameter 13.7% smaller than in healthy nursery trees. The most drastic reduction in tree growth was observed in the apple cultivar 'Golden Delicious' with a reduction of 64.4% in tree height and 42.9% in diameter. The apple cultivar 'Starkrimson' was the most resistant variety to artificial infection by ASGV showing no reduction in tree height or diameter. In Taiwan, ASGV was found to be the causal agent of a pear disease displaying symptoms of reduced size of foliage and leaf distortion. Two viral isolates were isolated from symptomatic pears. Sequence analysis of the cloned CP genes of these two isolates shared 88-92.4% nucleotide and 90.7-97.1% amino acid identities with those of ASGV isolates available in GenBank (Wu, 2010b).

In Japan, ASGV is one of the causative agents of apple topworking disease (Yanase , 1974, 1981) and induces decline syndrome in Mitsuba Kaido (*Malus sieboldii*) plants, which are used as rootstocks of apple trees in Japan (Yanase, 1981). ASGV has been reliably detected in varieties of citrus (ASGV synonym: CTLV) nectarine, plum, cherry and apricot (Kinard *et al*, 1996; Magome *et al*., 1997; Ito *et al*., 2002; Ito *et al*., 2003; Negi *et al*., 2010) as well as *Chenopodium quinoa* and *Nicotiana occidentalis* (James, 1998). In 2003, a new strain of ASGV was identified in kiwi fruit (*Actinidia chinensis*) imported from China. Leaves from these infected plants showed a variety of symptoms including interveinal mottling, chlorotic mosaics and ringspots. *Capillovirus*-like particles were observed under the electron microscope and were later confirmed to be ASGV with the ELISA technique. Sequencing results of this virus showed 95% amino acid identity with ASGV in the putative coat- and movement proteins. The morphological-, serological-, transmission-, and molecular characteristics of the virus from *Actinidia chinensis* were indistinguishable from those of ASGV. This was the first report of ASGV on a species of *Actinidia* (Clover *et al*., 2003).

Apple stem pitting virus is a latent virus present commonly in pome fruit trees worldwide. A study performed by Zhao *et al*. (2009) showed ASPV to be mainly distributed in the palisade tissue of mesophyll cells, the external cortex of the shoot tip, and its newly formed vascular bundles. A survey conducted in the major pome fruit growing regions of Greece showed ASPV to predominantly infect apple (91.8%) and pear (51.3%). ASPV was detected in

almost all cultivars and rootstocks tested for the survey (Mathioudakis *et al*., 2010). Apple stem pitting virus has also been reported to cause Pear vein yellow disease in pear (Jelkmann, 1994; Leone *et al*., 1998; Nemchinov *et al*., 1998; Rossini *et al*., 2010). Leone *et al*. (1995) evaluated symptoms on woody apple and pear indicators over a period of 4 years. This study showed evidence that the same virus isolates were able to cause syndromes related to ASPV and PVYV and thereby confirmed the identity of ASPV and PVYV as one virus that caused different syndromes in apple and pear. Sequence analysis of the CP gene of an isolate causing PVYV on a domestic pear cultivar in Taiwan showed 80.5-86.7% amino acid identity with the amino acid sequences of the CP gene products of 19 reported ASPV isolates (Wu *et al*., 2010a). Paunovic and Rankovic (1998) found evidence that allowed a preliminary identification of quince fruit deformation virus as a strain of PVYV and/or ASPV. Apple stem pitting virus has been reliably detected from leaf, bark and bud tissues of infected ASPV and/or PVYV apple and pear trees (Nemchinov *et al*, 1998).

Mixed infections of ASGV and ASPV often occur, usually in a complex with other latent pome fruit viruses (Nickel *et al*., 2001; Kundu, 2003; Caglayan *et al*., 2006). Caglayan *et al*. (2006) reported mixed infections of latent viruses of pome fruit samples tested with the most common being ASPV and *Apple chlorotic leaf spot virus* (ACLSV) (84.21%), followed by ASPV and ASGV (36.84%) and ACLSV and ASGV (26.32%). In 2004, a preliminary study was done in Albania to determine the presence of pome fruit viruses in 140 apple and 19 pear samples from 25 orchards. Although the infection rates for apples and pears were 100% and 84.2% respectively, the same viruses were present, but less frequently in pear. For apple, the prevailing viruses were ASPV (98.6%), ACLSV (97.8%), and ASGV (91.4%). In pear, the infection results were PVYV (87.5%), ACLSV (81.2%), and ASGV (12.5%) (Myrta *et al*., 2004).

If the high mixed infection rate of ASGV and ASPV was taken into account, these viruses have been reliably detected, separately or in combination with each other in host species throughout the world, including Africa (James, 1998; Youssef *et al*., 2010), Asia (James, 1998; Clover *et al*., 2003; Hirata, *et al*., 2003; Ito *et al*., 2003; Salem *et al*., 2005; Caglayan *et al*., 2006; Ismaeil, F., 2006; Birisik *et al*., 2008; Dhir *et al*., 2009; Ferretti *et al*., 2010; Gegi *et al*., 2010; Wu *et al*., 2010a; Wu *et al*., 2010b; Li and Yang, 2011; Liu *et al*., 2012; Liu *et al*., 2013), Australia (Rodoni and Constable, 2008), Europe (Varveri and Bem, 1995; Marinho *et* al., 1998; Nemchinov *et al*., 1998; Schwarz and Jelkman, 1998; Kummert *et al*., 2001; Kundu, 2001; Myrta *et al*., 2004; Salem *et al*., 2005; Mathioudakis *et al*., 2006; Barone *et al*., 2008; Hassan *et* al., 2008; Kundu, 2008; Ferretti *et al*., 2010; Gadiou *et al*., 2010; Mathioudakis *et al*., 2010; Pupola *et al*., 2011; Liebenberg *et al*., 2012), North America

(Kinard *et al*, 1996; James, 1998; Marinho *et al*., 1998) and South America (Nickel *et* al., 2001; Rossini *et al*., 2010).

2.4. Detection methods of ASGV and ASPV infection in pome fruit

Many cultivated fruit crops are maintained through vegetative propagation which makes the detection of viruses in fruit tree crops complicated since mixed infections of several viruses in one fruit tree is a common phenomenon (Nickel *et al*., 2001; Kundu, 2003; Caglayan *et al*., 2006). Plants used for propagation material and plants grown as part of a fruit production program are continuously visually inspected to diagnose viral diseases and afflictions. However, most commercially grown apple and pear varieties remain symptomless when infected by ASGV and ASPV. There are also no known vectors for ASGV and ASPV and both viruses appear to be transmitted mechanically or by the use of infected propagation material during grafting. Therefore, different methods other than visual inspection and identification should be used to detect ASGV and ASPV infection. Advancements in serological and molecular technologies have greatly improved the speed and specificity of virus identification. These methods vary in sensitivity of detection which affects the reliability of the specific method used.

2.4.1. Biological indexing

Currently the detection of ASGV and ASPV in apple trees for certification purposes, also in South Africa, are met by woody biological indexing which, in field testing, can take up to at least two years. Biological indexing consists of four steps (Figure 2.6):

1. Any appropriate virus free rootstock is used for biological indexing.
2. A test bud (bud of the variety tested) is grafted onto the rootstock during spring. The grafted test bud is securely wrapped with Plastrip to prevent dehydration and possible wound infection.
3. An indicator bud (specific for the disease tested) is grafted onto the rootstock above the test bud during spring. The grafted indicator bud is securely wrapped with Plastrip to prevent dehydration and possible wound infection.
4. After 21 days, the Plastrip is removed from the indicator bud allowing it to grow for symptom expression. Only the indicator bud is allowed to grow for symptom expression. If present, the virus spreads from the test bud into the plant and is expressed in the active growth of the indicator bud. Symptom expression is evaluated over a period of two active growing seasons.

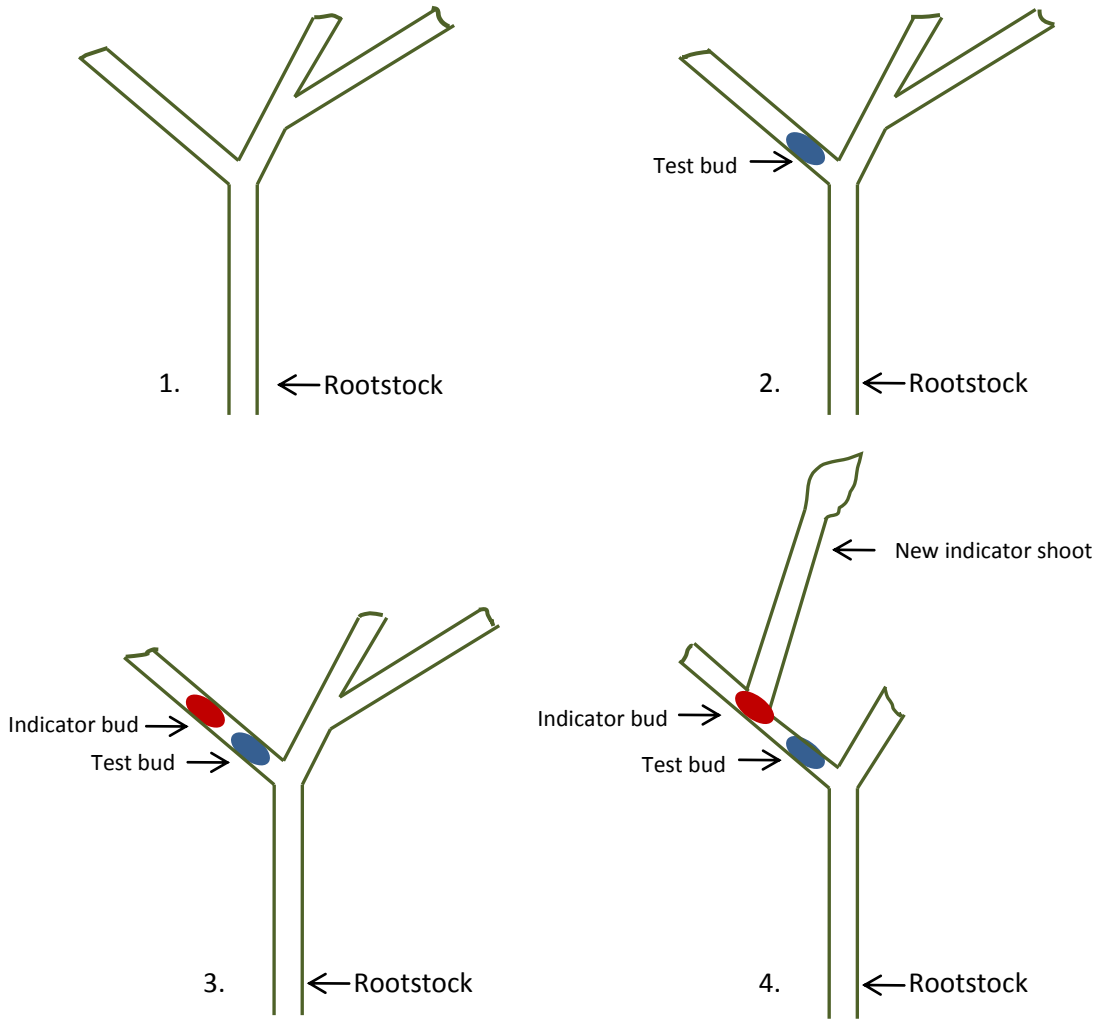

Figure 2.6. An illustration of biological indexing using a woody indicator for symptom expression. 1. An appropriate rootstock is chosen. 2. The test bud is crafted onto the rootstock. 3. The indicator bud is grafted onto the rootstock above the test bud. 4. Only the indicator bud is allowed to grow for symptom expression (Unpublished).

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

The history of the ELISA method dates back to the 1950s when radioimmunoassay (RIA), a technique using radioactively labeled antigen, was the only option for conducting an immunoassay. Radioimmunoassay was first described by Yalow and Berson in 1960 where it was used to measure endogenous human plasma insulin. From 1960 the application of the method expanded rapidly following the classical pattern of research – building on results published by colleagues in other fields. The first paper on ELISA as we know it today was published in 1971 by Engvall and Perlmann. It demonstrated quantitative measurement of IgG in rabbit serum with alkaline phosphatase as the reporter label.

The ELISA technique is very quick and simple to perform and incorporates the use of antigen absorbed to microplates or to antigen captured by unlabeled antibody absorbed to

microplates, and the detection thereof with enzyme-linked antibodies (Clark and Adams, 1977). Amongst the different types of ELISA methods used, the DAS-ELISA is the ELISA method that allows both the detection and the quantification of antigen (Figure 2.7) (Berg *et al*., 2002). With the DAS-ELISA method an antibody containing solution, which is specific to a particular antigen, is incubated in and absorbed to the wells of a plastic microtitre plate, which allows a small proportion of the antibody to coat the surfaces of the wells. A subsequent blocking step may be used to prevent non-specific binding of reagents added thereafter, although in some virus ELISA procedures a blocking step is not used. After unbound antibody has been washed away, samples of known or unknown antigen content are incubated in the antibody-coated wells in suitable buffers that do not allow non-specific binding of the antigen to the wells. If present, the antigen binds to the antibody. In the final step a second antibody (possibly of different origin) specific for the antigen, which is enzyme-linked (typically with enzymes such as calf intestine alkaline phosphatase or horse radish peroxidase), is added to the well. After incubation and washing, substrate (such as *p*-nitrophenylphosphate for alkaline phosphatase) containing solution is added to the wells causing an enzyme reaction and visual coloration in wells where the antigen was present. With this enzyme, the hydrolysis of the substrate releases *p*-nitrophenol which in turn changes the color of the reaction mixture from colorless to yellow. In the DAS-ELISA method, the amount of color formed is directly proportional to the amount of antigen present. This type of ELISA method permits the measurement of extremely small quantities of antigen in the nanogram to picogram range (Berg *et al*., 2002).

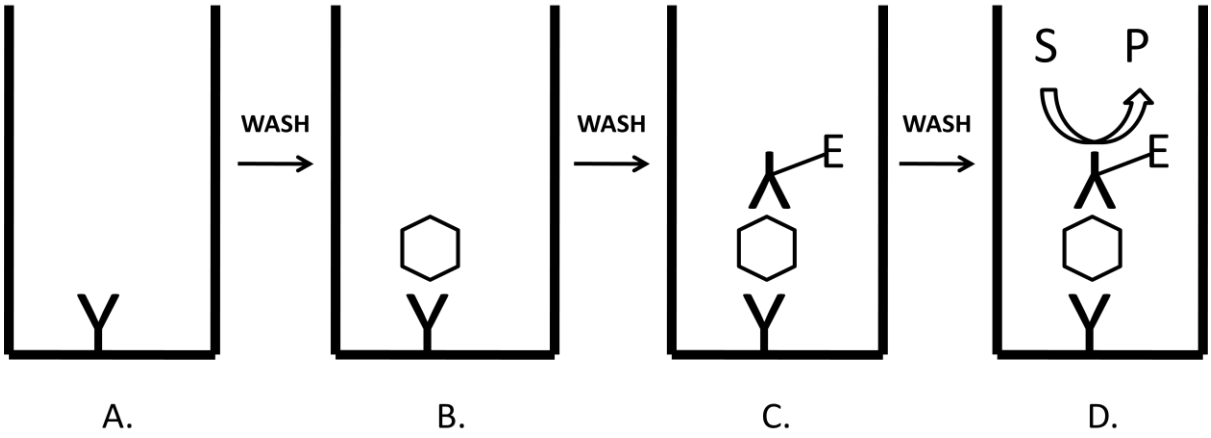

Figure 2.7. An illustration of the DAS- ELISA method. A. Specific antibody absorbed to plate. B. Specific antigen binds to antibody. C. A second enzyme-linked antibody binds to antigen. D. Substrate is added and converted by enzyme into a colored product. The amount of color formation is proportional to the amount of antigen present (Berg *et al*., 2002).

2.4.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

PCR was developed by Kary Mullis in 1983 (Mullis *et al*., 1986). The method involves DNA synthesis by unwinding DNA and using each strand of the parent molecule as a template to produce a complementary 'daughter' strand. Primers are synthetically produced DNA sequences usually around 20 nucleotides long that are complementary to the template sequence. Copying relies on the ability of nucleotides to base pair according to the Watson and Crick rules. The template strand therefore specifies the base sequence of the new complementary DNA strand (McPherson and Moller, 2006).

The PCR is comprised of three distinct steps, all governed by temperature (McPherson and Moller, 2006) (Figure 2.8):

- Denaturation: the double-stranded template DNA is denatured by heating, typically to 94°C, to separate the complementary single strands.
- Annealing: the reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template. The single strands of the template DNA are too long and complex to be able to re-anneal during this rapid cooling phase.
- DNA synthesis: the reaction is heated to a temperature typically 72°C for efficient DNA synthesis by thermo stable DNA polymerase.

Reverse transcriptase is an enzyme that can be used used to generate cDNA from a RNA template. This process is termed reverse transcription. PCR amplification can then be performed on the resulting cDNA. RT-PCR can be performed as a one-step or two-step protocol (Figure 2.8). The reverse transcriptase reaction can be performed on either total cytoplasmic RNA or purified mRNA (McPherson and Moller, 2006).

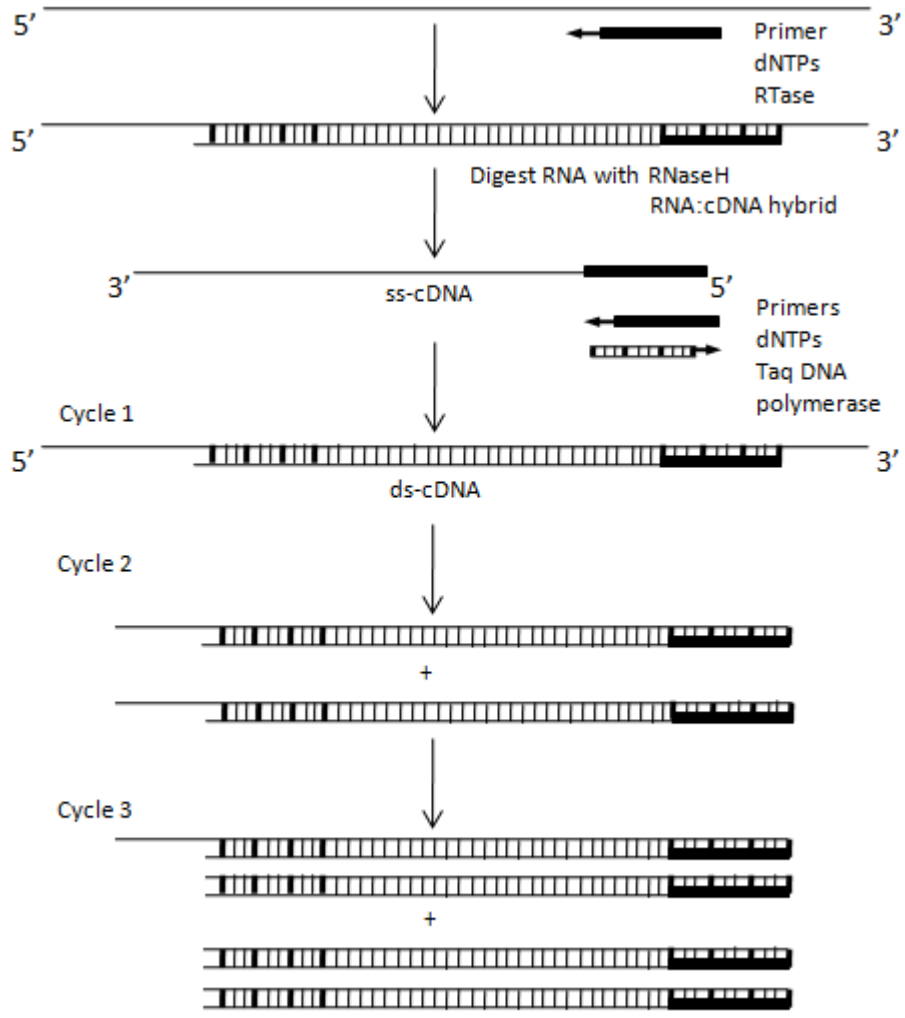

Figure 2.8. Diagrammatic representation of RT-PCR. Each cycle of PCR consists of denaturation, annealing and extention. RT = reverse transcriptase, ss- and ds-cDNA = single- and double stranded cDNA respectively. dNTPs – deoxynucleotide triphosphates.

2.5. A comparison of testing methods

Symptoms for ASPV can be diagnosed from species such as Spy 227- and Virginia Crab apple with biological indexing. A study done by Schwarz and Jelkmann (1998) showed biological indexing to be a very reliable method for the detection of ASPV from diseased isolates that induce symptoms on woody indicators two years after infection. A total of 24 isolates that showed epinasty and decline on Spy 227 tested positive for ASPV using primer combination ASPV9263 and ASPV9019 which generated an amplicon of 264 bp. During the same study, a total of 14 samples from pear propagated cultivar Williams and diseased with PVYV and/or other related diseases such as red mottle, quince sooty ring spot or quince fruit deformation, as well as nine leaf samples displaying stony pit were tested for ASPV using the same primer pair ASPV9263 and ASPV9019. An amplicon of 264 bp, identical in size to that obtained with ASPV was amplified from these isolates. Clear ASPV symptoms on

Virginia Crab and Spy 227 (apple indicators), that corresponds with PVYV symptoms on C 7/1 *Pyronia veitchii* (pear indicators), have confirmed the identity of ASPV and PVYV as one virus causing different syndromes in apple and pear (Leone *et al*., 1995; Leone *et al*., 1998).

In 1995, Howell and Mink screened different *Malus* clones for ASGV sensitivity and discovered a specific selection of *Malus micromalus* (GMAL 273) to be a highly sensitive, rapid reacting woody plant indicator for ASGV. GMAL 273 displayed distinctive foliage symptoms of ASGV within 14-21 days. This is a big contrast to the 6 to 8 months that is required for reliable first inspection ASGV symptoms to appear on Virginia crab indicators, grown under the same conditions, and 18 months to complete. This particular study also showed two individual selections of *M. yunnanensis* and *M. tschonoskii* to display particularly distinctive symptoms when inoculated with ASGV. However, these two clones required 40 days for strong symptom development compared to the 14 to 21 days of *M. micromalus* GMAL 273. A further study by Howell *et al*. (1996) screened Virginia crab apple and the three *Malus* selections over a period of two years with 19 isolates of ASGV from diverse geographical origins. Foliar ASGV symptoms were produced most consistently on *M. micromalus* GMAL 273 and least consistently on Virginia crab apple. *M. micromalus* was therefore found superior to Virginia crab as a rapid ASGV indicator on woody plants.

Biological indexing by grafting onto woody indicators can be described as a ‘baseline’ test for the certification of propagation material, but appears cumbersome, lengthy and expensive to perform taking into account the time, space and manpower required. It is also not in line with the actual constraints of producing certified material in the current agriculture industry where volume, distances and the rapidity of the exchange of material have raised dramatically. Historically methods such as biological indexing are replaced with methods that are less time consuming, more specific and more sensitive in virus detection.

Although ASPV is one of the most important latent viruses infecting apples and pears, the detection of ASPV by ELISA is inadequate due to the absence of good quality commercial antisera with regard to stability and sensitivity (Paunovic and Jevremovic, 2006). In 1997, a study was done to develop an immune-capture polymerase chain reaction and plate-trapped ELISA for the detection of ASPV. Isolates of ASPV and PVYV were sap transmitted from apple and pear cultivars to a reliable herbacious indicator *Nicotiana occidentalis* spp., *oblique* (Jelkmann and Keim-Konrad, 1997). However, nucleic acid extraction, including a proteinase K-digestion of leaf homogenates prior to chloroform/phenol extraction was of limited use for RNA template preparation. A fusion protein-specific antiserum 647 was prepared to *in vitro* expressed viral CP in order to improve RNA template preparation and the reliability of PCR tests from woody plant tissue. Only antiserum 647 reacted in an indirect

plate-trapped ELISA with ASPV isolates from *Nicotiana occidentalis.* Additionally, similar positive test results could be obtained for PVYV in pear but not in apple, proving this method to be non-satisfactory. Currently, antisera are mostly prepared using *in vitro* expressed viral CP gene. In 2011, Li and Yang tried to prepare a very sensitive and reliable antiserum to ASPV using *in silico* prediction of antigenic epitopes. Two linear synthetic peptides were prepared and conjugated with carrier protein. The antisera were obtained by immunizing rabbits. Only one of the designated antisera detected ASPV in apples.

In another attempt to produce antiserum against Apple stem pitting virus, CP (ASPV CP) from five ASPV isolates were amplified and cloned into bacteria and yeast expression vectors (Komorowska and Malinowski, 2009). The purified proteins used for immunization of rabbits produced antibodies that could be used for immunocapture RT-PCR detection of ASPV in woody tissue, but ELISA results were not satisfactory. Gugerli and Ramel (2004) successfully developed monoclonal antibodies (MAbs) to ASPV and PVYV for fast and large scale testing of apple and pear propagation material. These MAbs reacted well to a wide range of ASPV and PVYV isolates maintained on *Nicotiana occidentalis* as well as on apple and pear. Both viruses were well detected by ELISA in older and intermediated leaves of apple and pear. ASPV could also be detected in apple seeds. The MAbs also allowed the detection of ASPV and PVYV from crude extracts of woody scrapings of dormant material in winter. MAb based ELISA exceeded biological indexing, demonstrating that large scale detection of ASPV and PVYV with DAS-ELISA, based on these new MAbs, is possible. Although the South African Deciduous Fruit Plant Certification Scheme only prescribes biological indexing as the testing method for ASPV, DAS-ELISA kits for the ASPV is commercially available.

ELISA can be used to successfully detect ASGV in leaves of woody plants such as pome fruit during the active growing season when the virus is active within the plant (Wu *et al*., 1998; Corvo and Barros, 2001). However, when the virus is present in low concentrations in infected trees in orchards, i.e. summer or in dormant woody tissues, the ELISA is the inappropriate method to use. Leaves collected in spring during flowering are the most suitable tissue for virus detection using the ELISA technique. Leaves collected in summer or other tissues such as bark, dormant buds, and petals are not reliable material for ASGV detection by ELISA (Kundu *et al*., 2003).

Detectable amounts of ASGV are generally found in all material (bark, dormant buds, petals and leaves) during spring using RT-PCR. However, leaves collected in spring during flowering are the most suitable tissue for virus detection using the RT-PCR technique. (Kundu *et al*., 2003). ASGV have also been reliably detected in fruit and root tissues of

infected trees (Kinard *et al*, 1996). Sample tissue used for RT-PCR can be stored for more than 4 months at -80°C without it having an effect on the reliable detection of ASGV (James, 1998).

The RT-PCR assay allows accurate diagnosis of ASPV and its pear strain PVYV from leaf, bark and bud tissues of diseased apple and pear trees (Nemchinov *et al*, 1998; Paunovic and Jevremovic, 2004; Mathioudakis *et al*., 2010). Sequencing and hybridization analysis of these isolates revealed a potentially high level of molecular variability. A survey conducted in the major pome fruit growing regions of Greece showed ASPV to be predominantly in apple (91.8%) and pear (51.3%). ASPV was detected in almost all cultivars and rootstocks tested for the survey using RT-PCR assays amplifying part of the RNA-dependant RNA polymerase (RdRp) and the CP of the virus (Mathioudakis *et al*., 2010).

The RT-PCR appear to be more effective than ELISA when the virus is present in low concentration or when the detection is possible only from specific tissue or at specific periods from their woody tissue hosts. Research, comparing biological indexing, ELISA, multiplex PCR and RT-PCR showed multiplex PCR and RT-PCR to be a very reliable, method for ASGV and ASPV in pome fruit (Malinowski *et al*., 1998; Delano, 1999; James, 1999; Menzel *et al.*, 2002; Marinho *et al.*, 2003; Menzel *et al.*, 2003; Ma *et al.*, 2008; Komorowska *et al*., 2010). Menzel *et al.* (2003) found that infection of ASGV and ASPV by biological indexing could be confirmed by a single multiplex RT-PCR. This specific method can be reliably used in certification schemes for early and timely detection of ASPV and ASGV (James, 1998; Schwartz and Jelkman, 1998; Kundu, 2002; Paunovic and Jevremovic, 2008). However, additional infections of viruses other than ASGV and ASPV (using woody indicators) can be detected by multiplex RT-PCR, making it a much more sensitive method to use (Ito *et al*., 2002; Menzel *et al*., 2003). Although very challenging, the simultaneous detection of several viruses in a single PCR test reduces the cost of an analysis and the time needed. Instead of performing individual detection procedures, simultaneous detection reduces also the test time to that of a single determination.

In 2001, Klerks *et al*. developed a single tube AmpliDet RNA system for the rapid, gel-free detection of ASPV in apple tree tissues. This system relies on the specific amplification of viral RNA by nucleic acid sequence-based amplification and the simultaneous fluorescent detection of the amplification product through molecular beacons. The transcript RNA that was obtained by the ASPV-specific AmpliDet RNA showed sensitivity of a minimum of 100 molecules. This particular study also revealed that, compared to mechanical inoculation onto the ASPV indicator *Nicotiana occidentalis 37B*, only AmpliDet RNA consistently detected the virus in bark tissue. Other reliable season-specific sources for the detection of ASPV by

using the AmpliDet RNA system includes buds, petals, and fruits, but not leaves. In a continuously growing agricultural industry worldwide, reliable and rapid detection protocols for latent viruses is thus important in the implementation of sanitary control of propagation- and multiplication material of fruit trees.

2.6. Phylogenetic analysis

Phylogenetics is the study of evolutionary relationships whereas phylogenetic analysis is the means of inferring or estimating these relationships. The results of phylogenetic analyses are usually depicted as branching, treelike diagrams that represent an estimated pedigree of the inherited relationships among molecules, organisms or both. A clade represents a set of decendants from a single ancestor. Therefore, group members of a clade share a common evolutionary history and are more related to each other than to members of another group or clade. A given group is recognized by sharing unique features that were not present in distant ancestors. These shared, derived characteristics can be anything that can be observed and described e.g. two sequences having developed a mutation at a certain base pair of a gene. The following assumptions are made in phylogenetics (Brinkman and Leipe, 2001):

- Any group of organisms is related by descent from a common ancestor
- Change in characteristics occurs in lineages over time.

The resulting relationships from phylogenetic analysis are most commonly represented in a phylogenetic tree consisting of the following (Brinkman and Leipe, 2001):

- A clade – a monophyletic taxon. Clades are groups of organisms or genes that include the most recent common ancestor of all of its members and all of the decendants of that most recent common ancestor
- A taxon – any named group of organisms, but not necessarily a clade
- Branch lengths – correspondent to divergence
- A node – a bifurcating branch point.

Sequences within macromolecules have surpassed morphological characters as the most popular form of data for phylogenetic analysis. Although numerous phylogenetic algorithms, procedures and computer programs are available for phylogenetic analysis, their reliability and practicality are dependent on the structure and size of data (Brinkman and Leipe, 2001). Two popular character-based methods used for phylogenetic analysis include parsimony and maximum likelihood (Swofford *et al*., 1996; Cho, 2012). Besides the use of character data at all steps, these two methods have little in common with each other.

Phylogenetic analysis using parsimony uses an optimization criterion that adheres to the principle that the best explanation of the data is the simplest. There are several variants of the method that differ with regard to the permitted directionality of character state change (Swofford *et al*., 1996). Parsimony involves a site-by-site analysis of nucleotide sequence data. For each tree topology, it calculates the numbers of changes that are observed to calculate the observed site pattern. The number of changes is used to calculate a parsimony score for each tree topology. The topology with the smallest number of changes is taken as the estimate of the phylogeny which is known as the most parsimonious tree. However, analyses can find many parsimonious trees with the same tree length which represent alternative topologies. The method was initially proposed for gene-frequency data analysis and morphological character analysis (Edwards and Cavalli-Sforza, 1963; Cavalli-Sforza and Edwards, 1967; Camin and Sokal, 1965). There has been much controversy concerning the method since it does not make explicit assumptions using the evolutionary process (Felsenstein, 1973, 1978; Felsenstein and Sober, 1986; Yang, 1995; Whelan *et al*., 2001).

The process of constructing a phylogenetic tree using maximum likelihood involves finding the topology and branch lengths of the tree that will yield the highest probability of observing the DNA sequences in the data matrix and their relationship to each other (Cho, 2012). Maximum likelihood, therefore, searches for the evolutionary model, including the tree itself, which has the highest likelihood of producing the observed data, derived for each base position in an alignment. The likelihood is calculated in terms of the probability that the pattern of variation at a site would be produced by a particular substitution process, given a particular tree at the overall observed base frequencies. The likelihood becomes the sum of the probabilities of each possible reconstruction of substitutions under a particular substitution process (Brinkman and Leipe, 2001).

A study performed by Yang (1995) showed phylogenetic analysis using parsimony to be very efficient where a simple model of nucleotide substitution was assumed to generate data. The topology was as high as, or even higher than that of the maximum likelihood method. However, when the model became more complex and realistic, the probability for the parsimony method to recover the true topology of the model presented and its performance relative to that of the maximum likelihood method, generally deteriorated. However, the maximum likelihood method appeared to be preferable to parsimony since the complexity of the process of nucleotide substitution in sequences is well recognized. This was confirmed by Felsenstein (1973, 1978, 1988) who suggested that when the amount of evolution is small and the rate of evolution is more or less constant amongst lineages, parsimony may be an acceptable approximation to likelihood.

Phylogenetic analysis using parsimony produces many trees from which a strict consensus can be generated. Branch lengths (either actual characters or drawn on a proportional scale of the total matrix length) are indicated on the shortest trees from the parsimony analysis or on the most likely trees computed from the maximum likelihood analyses. Bootstrap support values are generated using bootstrapping procedures in the parsimony analysis to determine clade support. Phylogenetic analysis using maximum likelihood produces one single tree with bootstrap support values. Two different phylogenetic analyses, such as parsimony and maximum likelihood, are performed on the same data matrix to compare the resulting phylogenetic trees of each analysis. If similar results are obtained this indicates agreement between the two methods and therefore stronger conclusions about the evolutionary relationships between the different species and/or sequences can be made.

2.7. Virus testing within the South African Deciduous Fruit Plant Certification Scheme

In South Africa, the aim of the South African Deciduous Fruit Plant Certification Scheme and plant improvement organizations such as the South African Plant Improvement Organization (SAPO) Trust is to continuously strive to release and distribute optimal amounts of certified, disease-free material into the deciduous fruit industry. Pome fruit trees, including apples, pears and quince, are tested for the presence of viruses as prescribed by the South African Deciduous Fruit Plant Certification Scheme (http://www.saplant.co.za/spv.php).

Pome fruit trees are tested for ASGV and ASPV in a testing sequence depending on the classification of the origin of the material tested. Apple trees are also tested for ACLSV and *Apple mosaic virus* (ApMV). Trees from nucleus blocks (plants established from clean virus-free imported material) and foundation blocks (plants established from nucleus material) are tested annually for these viruses described within the South African Deciduous Fruit Plant Certification Scheme. Mother blocks are established from clean certified foundation block material. Since these pome fruit viruses are mechanically transmittable and the risk for secondary infection within mother blocks is extremely low, the prescribed testing sequence for mother blocks are once every 5 years. Should blocks be found to be infected with a specific virus, the plants they originated from are destroyed and a new, virus free block is established from nucleus and/or foundation block material, depending on where the infection occurred. Positive mother blocks are discarded from the certification scheme meaning no material can be issued from these blocks.

Currently, the methods described for pome fruit virus testing include biological indexing and DAS-ELISA for ASGV and only biological indexing for ASPV. In recent years, concern has arisen as to whether the current European imported DAS-ELISA kit used for ASGV is able to

detect all South African strains of ASGV. For pome fruit material to become a registered clone it has to go through the 2-3 year period of biological indexing for ASGV and ASPV which is extremely time consuming.

Latent pome fruit viruses such as ASGV and ASPV are viruses that show few if any symptoms and symptom expression can therefore not be used to reliably identify them. It is therefore extremely important for these viruses to be detected and thereby managed within the South African Deciduous Fruit Plant Certification Scheme. The optimal detection and identification of these viruses is therefore of outmost importance. Continuous research is needed to fulfill this aim by proposing a possible combination of methods, such as DAS-ELISA for routine detection, and RT-PCR for testing of mother material, which is extremely sensitive yet costly, to supply the needs of a rapidly growing industry.

Chapter 3. A comparison of the sensitivities of ELISA and RT-PCR in the detection of Apple stem grooving virus in South Africa.

3.1. Introduction

ASGV (genus *Capillovirus*) is known to infect pome fruits trees (including quince). Other hosts that have been documented to be infected with ASGV include varieties of citrus, nectarines, plum, cherry and apricot (Negi *et al*., 2010) as well as *Chenopodium quinoa* and *Nicotiana occidentalis* (James, 1998). In 2003, a new strain of ASGV was identified in kiwi fruit (*Actinidia chinensis*) imported from China (Clover *et al*., 2003).

Presently there is no known natural vector for the virus and it is assumed that the virus is only transmitted mechanically or by grafting. ASGV is very specific with regard to symptom expression and remains symptomless in most commercially grown pome fruit varieties. In susceptible varieties such as Virginia crab apple and a specific selection of *Malus micromalus* (GMAL 273), ASGV is known to cause stem grooving, brown line and graft union abnormalities. For this reason, Virginia crab apple and *Malus micromalus* (GMAL 273) are used worldwide as known indicators for biological indexing of ASGV to identify possible infection in varieties of which the ASGV status is unknown (Howell and Mink, 1995; Leone *et al*., 1998; Desvignes *et al*., 1999; Mathioudakis *et al*., 2006; Negi *et al*., 2010). Symptoms observed on kiwi fruit (*Actinidia chinensis*) imported from China that was confirmed to be caused by ASGV included interveinal mottling, chlorotic mosaics and ringspots of leaves (Clover *et al*., 2003). A study done by Maxim *et al*. (2004) showed ASGV to decrease the height and diameter of tree growth on some susceptible apple cultivars. In Japan, ASGV is the causative agent of apple topworking disease (Yanase, 1974; 1981) and induces decline syndrome in Mitsuba Kaido (*Malus sieboldii*) plants, which are used as rootstocks of apple trees in Japan (Yanase, 1981). In Taiwan, ASGV was found to be the causal agent of a pear disease displaying symptoms of reduced size of foliage and leaf distortion (Wu, 2010b). ASGV has also been reported to be the causal agent for PBNLS disease (Shim *et al*., 2004, Shim *et al*., 2006).

ASGV can be detected using biological indexing (Schwarz and Jelkmann, 1998). Biological indexing requires a period of two growing season and is therefore extremely time consuming and not ideal for the rapid, accurate determination of virus infection within a specific plant. The only means of accurate identification of latent viruses such as ASGV within a plant is by using laboratory methods such as DAS-ELISA (Wu *et al*., 1998; Corvo and Barros, 2001; Kundu *et al*., 2003) and RT-PCR (Kummert *et al*., 1998; James, 1999, Kundu *et al*., 2003; Marinho *et al.*, 2003). Additional infections of pome fruit viruses other than ASGV (such as

ASPV, ACLSV and ApMV) can be detected by multiplex RT-PCR, making it a much more sensitive method to use (Ito *et al*., 2002; Menzel *et al*., 2003). It also reduces the cost of an analysis and the time needed in comparison to performing individual detection procedures for the different viruses.

The ELISA technique was first introduced to plant virology by Clark and Adams (1977) and ever since, many variations of ELISA have been developed (Clark and Bar-Joseph, 1984; Van Regenmortel and Dubs, 1993). Due to its adaptability, sensitivity and cost effectiveness, ELISA is used in a wide range of situations, especially to test large numbers of samples in a relatively short period of time. In comparison to the ELISA method, the sensitivity of nucleic acid-based detection systems was greatly improved by the development of the PCR method (Mullis *et al*., 1986). Many comparisons of the DAS-ELISA and RT-PCR methods for the detection of ASGV have been performed in different regions of the world including Spain (Kummert *et al*., 1998), United Kingdom (James, 1999), Portugal (Corvo and Barros, 2001) Hungry (Menzel *et al*., 2002; Marinho *et al*., 2003; Menzel *et al*., 2003) and Japan (Wu *et al*., 1998). All authors reported that both ELISA and/or RT-PCR can be used to successfully detect ASGV in leaves of pome fruit trees. A study done by Caglayan *et al*. (2006) showed that the number of plants identified to be infected by any pome fruit virus was higher when using RT-PCR compared to ELISA. From a total of 174 plants, 8.6% more samples were positive for pome fruit viruses, including ASGV, using RT-PCR in comparison to ELISA.

In this study, the detection limits of two testing methods, DAS-ELISA and RT-PCR, were compared for the detection ASGV in pome fruit material.

3.2. Materials and methods

3.2.1. Sampling

A total of 15 pome fruit trees that were infected with ASGV were selected for this study. These infected plants were maintained in a greenhouse at the SAPO Trust facility in Stellenbosch. Trees did not show any symptoms such as stem grooving, brown line- and/or graft union abnormalities. A standard procedure for annual seasonal virus testing was followed. From each tree a total of 10 leaves were randomly sampled from new growth across the tree.

3.2.2. Sample collection and preparation

A total of 15 plant samples were collected from the ASGV infected pome fruit collection maintained at the SAPO Trust facility, located on the Fleurbaix farm, Stellenbosch, South Africa. None of the plants showed ASGV associated symptoms. When comparing two

diagnostic methods such as DAS-ELISA and RT-PCR it is important to use uniformity throughout the whole process to ensure the validity of the results obtained. Thus the leaves of each sample were stacked onto each other and halved vertically using a clean sterile scalpel blade. One vertical half was used uniformly throughout the study for DAS-ELISA testing (left side) and one half for RT-PCR testing (right side). Each half was then halved horizontally and the upper and lower halves were tested separately and in duplicate. Leaves for sample preparation were cut around the middle point of the leaves to ensure optimal comparison of the different sub-samples (Figure 3.1).

3.2.3. Enzyme-linked immunosorbent assay (ELISA)

3.2.3.1. Sample and control preparation

For ELISA testing the Bioreba kit (Bioreba, Switzerland) was used according to the manufacturer's protocol. A total of 15 samples were prepared in duplicate. Using sterile scissors, each sample was cut into two representative samples (Figure 3.1) of which 1 g of fresh leaf sample of each sub-sample was macerated and homogenised in Bioreba general extraction buffer in the ratio 1:10 to have a total sample volume of approximately 10 ml. The ELISA protocol was also performed in duplicate as technical repeats. Macerated samples were incubated for 1 hour at room temperature before they were used.

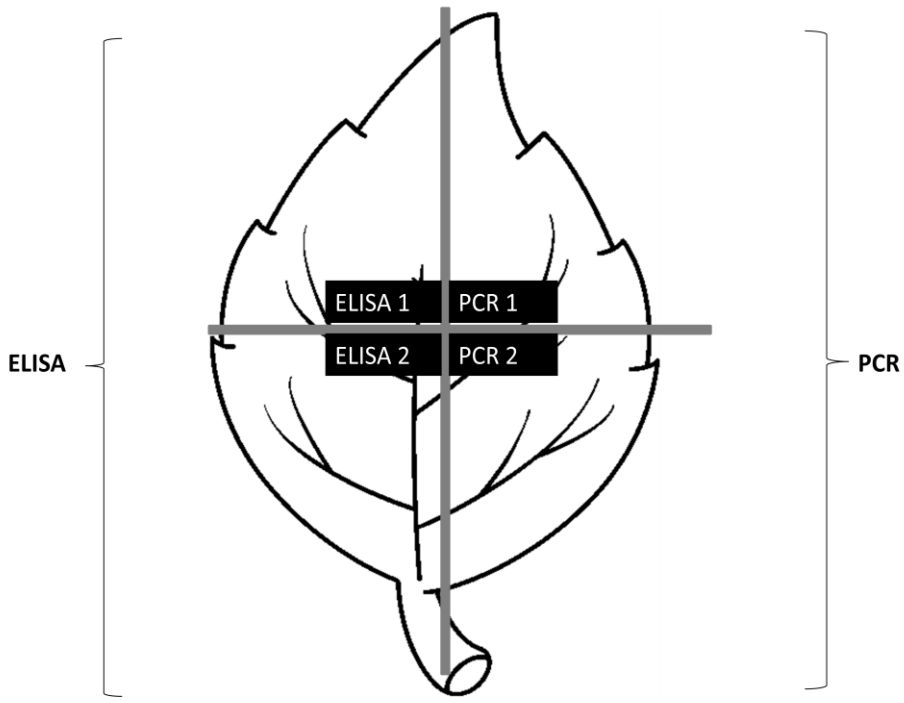

Figure 3.1. A schematic representation of sample preparation for the the ELISA- and PCR methods. For both methods each sample was halved horizontally and tested separately and in duplicate

A total of five uninfected pome fruit trees were selected as negative controls and were included on each ELISA plate. The same controls were used on all plates. A total of 1 g of fresh leaves of each negative control was macerated and homogenised in Bioreba general extraction buffer in the ratio 1:10 to give a total sample volume of approximately 10 ml.

3.2.3.2. Identification of ASGV using ELISA

For the coating step, IgG was diluted 1000x (1 µl IgG per ml coating buffer) after which 200 µl was added to each well of the Nunc Maxisorp microtiter ELISA plate (Thermo Scientific, Denmark). Plates were covered and incubated in a humid box for five hours at 30°C or overnight at 4°C. The wells were emptied, washed 3 times with 3 minute intervals between each wash using washing buffer. After the last wash interval, plates were patted dry on paper towels after which 200 µl of each separate homogenized sample (see sample preparation) was added to the wells of the coated microtiter ELISA plate. Samples were diluted and loaded to the wells in the following concentrations: undiluted, 1/2, 1/5, 1/10 and 1/25. Sap from an uninfected pome fruit tree was used to make dilution series mixtures. For this purpose, 5 g of uninfected sample was macerated and homogenised in Bioreba general extraction buffer in the ratio 1:10 to yield a total sample volume of 50 ml for use in the dilution series. Each sample dilution series was loaded in duplicate on two separate ELISA plates. The history of each sample was documented on a corresponding plate layout to ensure traceability between samples loaded and sample results. A commercial positive control (Bioreba, Switzerland) was added to each plate. A total of 5 negative controls were added to the plate to determine ELISA plate positive cut-off values. Plates were covered and incubated in a humid box overnight at 4°C.

After incubation, the wells were emptied and washed three times with three minute intervals between each wash using washing buffer after which they were patted dry on paper towel. Enzyme conjugate was diluted 1000x in conjugate buffer and 200 µl was added to each well of the microtiter ELISA plate. Plates were covered and incubated in a humid box for five hours at 30°C. The wells were emptied and subsequently washed as before. One mg/ml p-nitrophenyl was dissolved in substrate buffer after which 200 µl was added to each well of the microtiter ELISA plate. Plates were incubated for one hour at room temperature (18°C-25°C) in a dark room. Absorbance were measured after 1 hour with a spectrophotometer at 405 nm. Positive values were calculated as prescribed by the kit manufacturer (Bioreba, Switzerland):

(mean of 5 negative controls) + 3 x (standard deviance of 5 negative controls) + 10%

All sample readings higher than this value were considered positive.

3.2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

3.2.4.1. Sample preparation

For RT-PCR testing, the RNA of the same 15 samples that were used for ELISA testing was isolated. For RNA extraction, the RNeasy Plant Mini Kit (Qiagen, Germany) was used. A total of 0.1 g of fresh leaf sample in duplicate was used for each sample for RNA extraction. RNA was extracted as described by the manufacturer and eluted in a final volume of 40 µl. Extracted RNA from uninfected pome fruit material was used as a negative control.

3.2.4.2. Primer pairs

The primer pairs described by Menzel *et al*. (2002) were used in this study (Table 3.1). The cycling parameters were: 48°C for 30 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds and extention at 72°C for 60 seconds. This was followed by a final elongation step at 72°C for 10 minutes. The amplicon of the primer set is 273 bp corresponding to position 6039-6064 (sense) and 6286-6311 (antisense) of ASGV reference accession number AB004063 of the National Center for Biotechnology Information as described by Menzel *et al*. (2002).

Table 3.1. Primer sequence and expected size for ASGV detection used in this study.

Primer	**Primer sequence in 5'-3' orientation**	**Primer position**	**Product size**	**Reference**
Apple stem grooving virus				
Sense	GCCACTTCTAGGCAGAACTCTTTGAA	1474-1499	273bp	(Menzel *et al.*, 2002)
Antisense	AACCCCTTTTTGTCCTTCAGTACGAA	1711-1735		

3.2.4.3. Amplification of virus nucleic acid by RT-PCR

RNA samples were diluted and tested in duplicate in two different reaction mixtures in the following dilutions: undiluted, 1/2, 1/5, 1/10 and 1/50. Extracted RNA from an uninfected pome fruit tree was used to make the dilution series mixtures. The RT-PCR mixture contained 2.5 µl 10x PCR buffer (Bioline, UK), 1.25 µl of 0.1 M DTT, 1 µl of 25 mM $MgCl_2$, 1 µl ASGV forward primer (20 µM), 1µl ASGV reverse primer (20 µM), 1µl of 5 mM dNTP's (Bioline, UK), 0.25 µl of 5 U/µl Taq™ DNA polymerase (Bioline, UK), 0.125 µl of 200 U/µl SuperScriptTM III reverse transcriptase (Invitrogen, USA) and 14.875 µl Milli-Q® water. A

total volume of 23 µl sample mixture was used together with 2 µl of sample RNA to have a total reaction volume of 25 µl. All 15 samples were tested in duplicate at a sample dilution higher than the highest dilution that could detect ASGV with DAS-ELISA overall.

3.2.4.4. Gel electrophoresis

A 1% (w/v) agarose gel was prepared with 1 × TAE buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and Milli-Q® water. Ethidium Bromide (Sigma, USA) was added to visualize products under UV light. The electrophoresis buffer consisted of 1 × TAE buffer. Ten microliters of the PCR reaction mixture were mixed with loading buffer (Bioline, UK) and loaded on the agarose gel. Electrophoresis was performed at 100 V for 80-100 minutes using a power source (Cleaver Scientific, UK). A 100 bp DNA size marker ladder (Bioline, UK) was used to determine the size of amplified DNA fragments. The DNA bands were visualized under a UV transilluminator and portable darkroom (Cleaver Scientific, UK). Detection was regarded as successful where visual bands of the anticipated fragment length were observed.

3.2.5. Statistical analysis of DAS-ELISA and RT-PCR comparative results

In order to compare the sensitivity of the ELISA and RT-PCR methods, a formal statistical hypothesis test was used. A first option in this regard is Friedman's rank test, a two-way analysis of variance by ranks. The Friedman test statistic is given by $F = \frac{12}{Nk(k+1)} \sum_{j=1}^{k} R_j^2 - 3N(k+1)$, where N denotes the number of sample units, k is the number of treatments and R_j is the total rank for treatment j, $j = 1,\ldots,k$ (Siegel, 1956).

A rank order value of 1 was indicated for each sample of the method which detected the highest dilution of virus, and a rank order value of 2 was indicated to the method which detected a lower dilution of the sample. If both methods detected the virus at the same dilution, a value of 1.5 was indicated in both columns. The null hypothesis of no difference between the two methods was tested and a p-value was obtained.

The DAS-ELISA and RT-PCR results obtained were listed in columns in an Excel spreadsheet showing the rank order of whether the virus was detected by each of the methods or only one. The factor whereby one method was more sensitive than the other was calculated for each sample replicate, i.e where RT-PCR was more sensitive than DAS-ELISA, the factor was calculated by dividing the RT-PCR equivalent value of the highest dilution detected by RT-PCR by the DAS-ELISA value of the highest dilution detected by

DAS-ELISA for that specific sample. The geometric mean of the factors was then calculated in order to express the differences between the sensitivities of the two methods numerically.

To compare the sensitivities of the DAS-ELISA and RT-PCR, the difference in the relative quantities used were calculated. In the RT-PCR, 100 mg of leaf material was used for RNA extraction of which the isolated RNA was made up in 40 µl water provided by the manufacturer. Of this RNA solution, 2 µl was used in the RT-PCR with final volume of 25 µl, and all was loaded onto the gel for detection. The equivalent amount of plant material used was calculated as follows:

2 µl/40 µl x 100 mg ≡ 5 mg per reaction sample

(i.e. should a positive result be obtained. This reflects the presence of the virus in an amount of 5 mg of undiluted plant sample).

In the DAS-ELISA, 1 g of leaf material was homogenized in 10 ml of buffer of which 200 µl was used in the DAS-ELISA. The equivalent amount of plant material used was calculated as follows:

200 µl/10 000 µl x 1 000 mg = 20 mg per well

(i.e. should a positive result be obtained, this reflects the presence of the virus in an amount of 20 mg of undiluted plant sample). Thus the RT-PCR determination is based on a RNA equivalent virus level in 5 mg of plant material compared to 20 mg in the case of DAS-ELISA.

3.3. Results

3.3.1. DAS-ELISA detection of ASGV

The results of the calculated positive cut-off values for each of the five different ELISA plates used for ELISA testing are shown in Table 3.2.

Table 3.2. Results of negative controls of ELISA plates ASGV1-5 used to calculate the positive cut-off value calculation. NC = Negative control; STD = Standard deviance

Plate	NC1	NC2	NC3	NC4	NC5	Mean (NC)	3 x STD (NC)	Positive cut-off value
ASGV1	0.114	0.109	0.109	0.114	0.110	0.1112	0.0069	0.130
ASGV2	0.119	0.111	0.112	0.119	0.113	0.1148	0.0105	0.138
ASGV3	0.125	0.124	0.123	0.135	0.123	0.1260	0.0137	0.154
ASGV4	0.125	0.125	0.120	0.134	0.119	0.1246	0.0159	0.155
ASGV5	0.134	0.134	0.124	0.134	0.125	0.1302	0.0140	0.159

Table 3.3. Absorbance readings of samples at different dilutions after 1 h incubation and read at 405 nm. Samples giving a reading below the detection limit are indicated in blue.

Sample	Plate number	Positive cut-off value	Dilution series ELISA reading (highest reading from 2 biological controls and 2 technical controls in duplicate)				
			1/1	1/2	1/5	1/10	1/25
A1	ASGV1	0.130	0.308	0.191	0.153	0.140	0.124
F5	ASGV2	0.138	0.382	0.369	0.240	0.187	0.134
H7	ASGV2	0.138	0.757	0.376	0.199	0.153	0.136
J8	ASGV2	0.138	0.143	0.127	0.122	0.126	0.125
N10	ASGV3	0.154	0.223	0.144	0.131	0.129	0.130
G11	ASGV3	0.154	0.165	0.135	0.127	0.124	0.128
O12	ASGV3	0.154	0.386	0.215	0.165	0.146	0.140
P13	ASGV3	0.154	0.153	0.129	0.127	0.127	0.128
Q14	ASGV4	0.155	0.251	0.167	0.139	0.138	0.130
R 15	ASGV4	0.155	0.148	0.129	0.124	0.126	0.122
S17	ASGV4	0.155	0.545	0.260	0.169	0.146	0.132
T18	ASGV5	0.159	0.332	0.198	0.161	0.153	0.132
U19	ASGV5	0.159	0.233	0.158	0.138	0.131	0.129
W20	ASGV5	0.159	0.981	0.305	0.179	0.138	0.131
AA21	ASGV5	0.159	0.172	0.135	0.130	0.129	0.129

The positive status of 13 of the 15 samples could be confirmed for undiluted samples using the positive cut-off values calculated for each individual ELISA plate (see Table 3.2 and Table 3.3). Table 3.3 also indicated the DAS-ELISA readings of each sample at the different dilutions. Samples indicated in blue showed readings below the positive cut-off value.

3.3.2. RT-PCR detection of ASGV in comparison with DAS-ELISA

Figure 3.2 shows the results of the one repeat of the RT-PCR performed on all 15 samples at a dilution of 1:50. This dilution was higher than the highest dilution of 1:10 in which ASGV could be detected in DAS-ELISA for samples A1, F5 and H7 (see Table 3.3). All samples showed bands of approximately 273 bp under the UV transilluminator and were regarded as positive for the presence of ASGV. Samples that showed low virus titer with DAS-ELISA also showed RT-PCR bands with low intensity (e.g. sample P13, Lane 2) in comparison to samples that showed high virus titer with DAS-ELISA and the corresponding high intensity RT-PCR bands (e.g. sample O12, Lane 8).

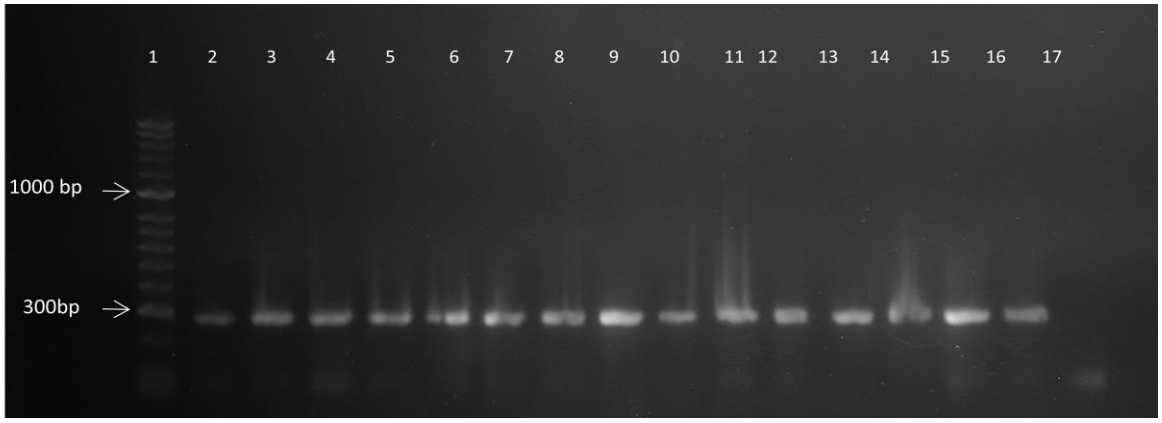

Figure 3.2. An image of the 15 RT-PCR products of one repeat listed in Table 3.2 at a dilution of 1/50. Lane 1:100 bp Hyperladder^{TM} II; Lane 2: Sample P13. Lane 3: Sample F5. Lane 4: Sample A1. Lane 5: Sample H7. Lane 6: Sample N10. Lane 7: Sample G11.Lane 8: Sample O12. Lane 9: Sample Q14. Lane 10: Sample J8. Lane 11: Sample R15. Lane 12: Sample S17. Lane 13: Sample T18. Lane 14: Sample U19. Lane 15: Sample W20. Lane 16: Sample AA21. Lane 17: Negative control.

Figure 3.3A and B show the results of 4 samples at a dilution of 1:50. Each sample was prepared and tested in duplicate in two different reaction mixtures. Results of reaction mixture 1 and 2 are represented by Figure 3.3A and 3.3B respectively. Table 3.4 show the DAS-ELISA detection limits of the 15 samples and their repeats compared to RT-PCR. It also shows the highest dilution at which samples could be detected using DAS-ELISA versus a RT-PCR dilution comparison of 1/50 which is higher than the highest dilution overall detected by DAS-ELISA. The factor whereby the one method is more sensitive than the other is indicated for each individual sample repeat.

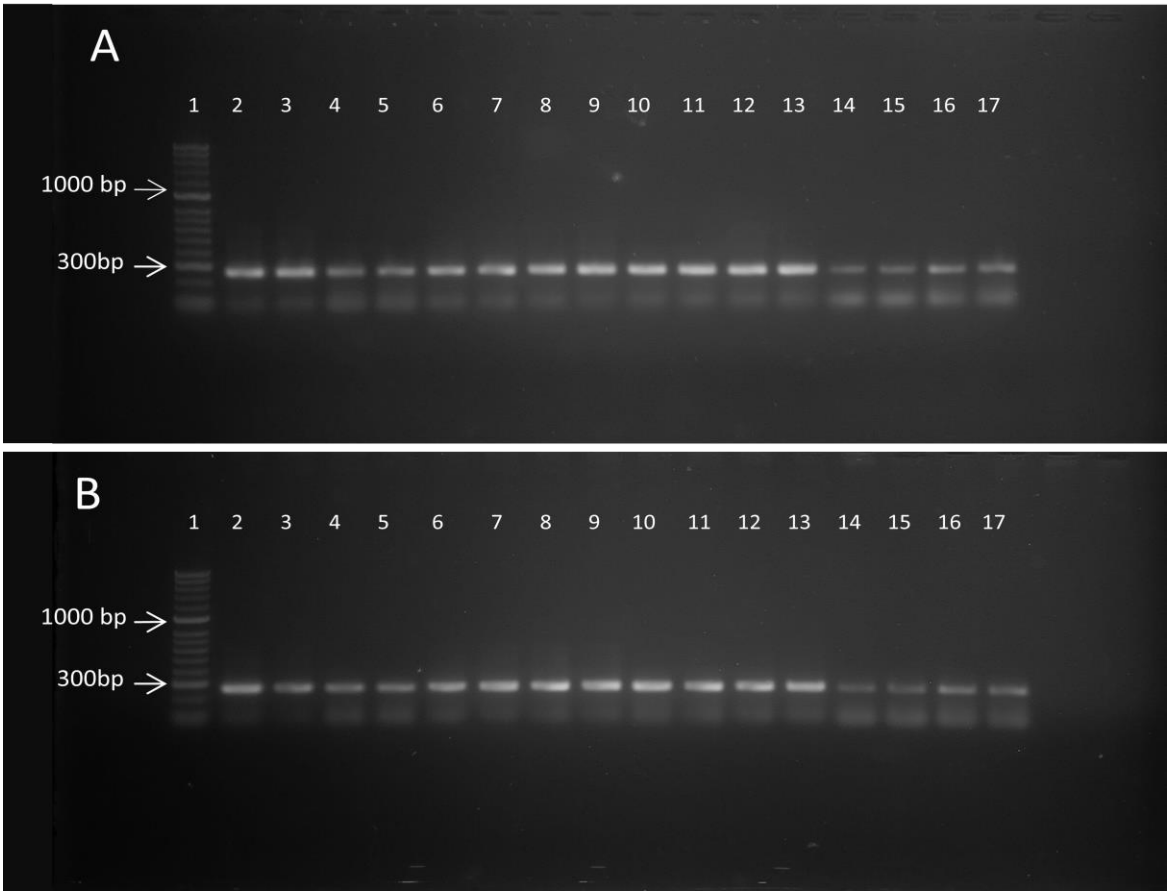

Figure 3.3A and 3.3B. PCR results of the similarities between the amplified RT-PCR products in a dilution of 1/50 of 4 samples prepared and tested in duplicate in two different RT-PCR mixtures. The dilution of 1/50 was chosen as the concentration higher than the highest concentration of ASGV that could be detected using DAS-ELISA (Table 3.4). Lane 1: Bioline 100bp Hyperladder^{TM}II. Lane 2-5:J8. Lane 6-9: S17. Lane 10-13: T18. Lane 14-17: P13.

The results obtained from duplicate DAS-ELISA versus RT-PCR at different dilutions are indicated in Table 3.4. The highest dilution of sample in which ASGV was detected with DAS-ELISA is indicated. RT-PCR results at a dilution of 1/50 for all 15 samples, a dilution higher than the highest dilution that could be detected with DAS-ELISA, are also indicated. The geometric mean of the factors listed in Table 3.4 was calculated as $10^{2.1}$ X. Since a standard dilution of 1/50 was used for all samples, this geometric mean indicated that RT-PCR is at least 126 fold more sensitive than the DAS-ELISA.

Table 3.4 Results of the different repeats per plant sample for the detection of ASGV by DAS-ELISA and RT-PCR. The highest dilution of sample detected with DAS-ELISA is indicated. All samples were tested with RT-PCR in a dilution higher than the highest dilution of sample detected with DAS-ELISA overall. The factor was calculated by dividing the RT-PCR equivalent value by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Biological repeat	Technical repeat	DAS-ELISA dilution optimal detection	RT-PCR dilution optimal detection	RT-PCR equivalent value to DAS-ELISA	Factor	Factor converted to a log value
8	A1.1	A1.1.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		A1.1.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
	A1.2	A1.2.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		A1.2.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
F5	F5.1	F5.1.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		F5.1.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
	F5.2	F5.2.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		F5.2.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
H7	H7.1	H7.1.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		H7.1.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
	H7.2	H7.2.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		H7.2.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
J8	J8.1	J8.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		J8.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	J8.2	J8.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		J8.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
N10	N10.1	N10.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		N10.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	N10.2	N10.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		N10.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
G11	G11.1	G11.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		G11.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	G11.2	G11.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		G11.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
O12	O12.1	O12.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		O12.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	O12.2	O12.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		O12.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X

Sample	Biological repeat	Technical repeat	DAS-ELISA dilution optimal detection	RT-PCR dilution optimal detection	RT-PCR equivalent value to DAS-ELISA	Factor	Factor converted to a log value
P13	P13.1	P13.1.1	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
		P13.1.2	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
	P13.2	P13.2.1	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
		P13.2.2	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
Q14	Q14.1	Q14.1.1	1/2	1/50	1/200	100 X	$10^{2.0}$ X
		Q14.1.2	1/2	1/50	1/200	100 X	$10^{2.0}$ X
	Q14.2	Q14.2.1	1/2	1/50	1/200	100 X	$10^{2.0}$ X
		Q14.2.2	1/2	1/50	1/200	100 X	$10^{2.0}$ X
R15	R15.1	R15.1.1	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
		R15.1.2	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
	R15.2	R15.2.1	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
		R15.2.2	Not detected	1/50	1/200	200 X	$10^{2.3}$ X
S17	S17.1	S17.1.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		S17.1.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
	S17.2	S17.2.1	1/10	1/50	1/200	20 X	$10^{1.3}$ X
		S17.2.2	1/10	1/50	1/200	20 X	$10^{1.3}$ X
T18	T18.1	T18.1.1	1/2	1/50	1/200	100 X	$10^{2.0}$ X
		T18.1.2	1/2	1/50	1/200	100 X	$10^{2.0}$ X
	T18.2	T18.2.1	1/2	1/50	1/200	100 X	$10^{2.0}$ X
		T18.2.2	1/2	1/50	1/200	100 X	$10^{2.0}$ X
U19	U19.1	U19.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		U19.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	U19.2	U19.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		U19.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
W20	W20.1	W20.1.1	1/5	1/50	1/200	50 X	$10^{1.7}$ X
		W20.1.2	1/5	1/50	1/200	50 X	$10^{1.7}$ X
	W20.2	W20.2.1	1/5	1/50	1/200	50 X	$10^{1.7}$ X
		W20.2.2	1/5	1/50	1/200	50 X	$10^{1.7}$ X
AA21	AA21.1	AA21.1.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		AA21.1.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X
	AA21.2	AA21.2.1	1/1	1/50	1/200	200 X	$10^{2.3}$ X
		AA21.2.2	1/1	1/50	1/200	200 X	$10^{2.3}$ X

3.3.4 Statistical analysis: DAS-ELISA and RT-PCR detection of ASGV using the Friedman test

The results of the determinations of DAS-ELISA versus RT-PCR were analysed using the Friedman test (Table 3.5). The null hypothesis of no difference between the two methods was tested and a p-value was obtained. Using the χ^2-approximation for the distribution of F under the null hypothesis with $F = 15$, the p-value from a χ_1^2-distribution corresponding to the observed value $F = 15$ is 0.0001075. The results show that the null hypothesis of no difference between the two methods can be rejected with a very high degree of confidence.

Table 3.5. The rank order of the 15 samples used to compare the sensitivity of DAS-ELISA versus RT-PCR. A rank order value of 1 was indicated for each sample in the column of the method which detected the highest dilution of virus. A rank order value of 2 was indicated to the method which detected a lower dilution of the sample. If both methods detected the virus at the same dilution, a value of 1.5 was indicated in both columns

Sample unit	Sample	RT-PCR	DAS-ELISA
1	A1	1	2
2	F5	1	2
3	H7	1	2
4	J8	1	2
5	N10	1	2
6	G11	1	2
7	O12	1	2
8	P13	1	2
9	Q14	1	2
10	R15	1	2
11	S17	1	2
12	T18	1	2
13	19	1	2
14	W20	1	2
15	AA21	1	2

3.4. Discussion

The DAS-ELISA kit from a well-known supplier (Bioreba, Switzerland) was successful in the detection of ASGV. ASGV could be detected in 13 of the 15 samples using the DAS-ELISA. Samples P13 and R15 showed absorbance readings at 405 nm that were lower than the positive plate cut-off value that was determined using the formula supplied by the kit supplier. The highest dilution that gave a positive reading with DAS-ELISA was 1/10

(samples A1, F5, H7 and S17). The success of DAS-ELISA is influenced by factors such as the sample material used, the structure and stability of the virus particle as well as the amount of virus particles present in a homogenized sample. The ASGV virion is a helically constructed filamentous particle of approximately 640 nm long and 12 nm in diameter (De Sequeira and Lister, 1969). This long virus structure may result in an unstable, macerated DAS-ELISA extraction mix, meaning the virus might partially break down before DAS-ELISA antibodies can bind.

ASGV could be detected in all 15 samples with RT-PCR at a dilution of 1/50. Thus RT-PCR was found to be at least 126 fold more sensitive than the DAS-ELISA for the detection of ASGV. Although leaves collected in spring during flowering are the most suitable tissue for virus detection using the DAS-ELISA and RT-PCR technique, detectable amounts of ASGV have been found in all plant material including bark, dormant buds, petals and leaves during spring using RT-PCR. Apart from the wide range of material used for virus detection, material stored at -80°C for more than 4 months can still be used to reliably detect ASGV with RT-PCR (James, 1999; Kundu *et al*., 2003). Storage of samples may also influence the stability of a specific virus. Therefore, for the purpose of this study, only fresh leaf samples during peak testing season were used to promote high levels of virus particles in samples. At low titers, DAS-ELISA may not be able to detect viral infection. This may be the reason why ASGV infection could not be confirmed in samples P13 and R15 using DAS-ELISA, even though this study was performed during spring when the highest possible virus levels are present in pome fruit trees. Viruses are known to be unevenly spread and distributed in many host plants and seeds. For this specific reason, leaves were sampled randomly from fresh growth to represent the status of the tree as a whole and were prepared uniformly to allow a valid comparison of the results obtained from both DAS-ELISA and RT-PCR.

Numerous comparisons of the sensitivities of the ELISA and RT-PCR methods for ASGV detection have been made (Wu *et al*., 1998; Corvo and Barros, 2001; Menzel *et al*., 2002; Marinho *et al*., 2003; Caglayan *et al*., 2006; Paunovic and Jevremovic, 2008). In 2006, a study performed by Caglayan *et al*. showed that ASGV could be detected in 8.6% more samples using RT-PCR than with DAS-ELISA. Hassan and Polak (2008) were able to confirm the virus status of samples that tested positive with ELISA with RT-PCR. However, RT-PCR revealed more infected trees than were confirmed with ELISA. Furthermore, Candresse *et al*. (1995) found that in comparison to DAS-ELISA, RT-PCR detects lower levels of viral titer and can therefore be used as a diagnostic tool for the detection of ASGV over longer periods (not limited to spring only). The fact that ASGV was not detected in this study in two samples by DAS-ELISA, but ASGV was detected by RT-PCR in all 15 samples, shows that RT-PCR has distinct advantages when virus levels in plant material are low. In

seasonal routine laboratory tests where the DAS-ELISA is the only compulsory testing method prescribed for virus detection, samples with low virus levels might show false negative results.

In a fast growing industry in which new varieties are continuously released, the rapid and reliable detection of the plant virus status of pome fruit in South Africa is of utmost importance. Since viruses have a huge impact on the quality of the tree, tree growth and fruit yield, it is also important to identify viruses, if present, in the early stages of the propagation process. Virus infected material can thereby be eliminated from the system before the establishment of mother blocks. The risk of economic losses due to blocks that have been discarded from the certification system will therefore be lower.

Worldwide DAS-ELISA is used for routine testing within certification schemes. Compared to RT-PCR, DAS-ELISA is more cost effective than RT-PCR and has a higher throughput of samples. Although RT-PCR is a more sensitive method, it is more expensive and has a lower sample throughput than DAS-ELISA. However, despite the disadvantages of higher cost and lower throughput, the increased sensitivity of RT-PCR may outweigh the advantages of DAS-ELISA for the detection of ASGV in the South African Deciduous Fruit Plant Certification Scheme.

Chapter 4. The determination of the genetic variation of Apple stem grooving virus in South African pome fruit orchards based on CP gene sequences

4.1. Introduction

ASGV belongs to the family Flexiviridae *(*genus *Capillovirus)* and is distributed worldwide wherever pome fruit is cultivated (James, 1998; Mathioudakis *et al*., 2006; Negi *et al*., 2010). Pome fruit varieties in South Africa include varieties that have been bred locally, as well as varieties imported from different countries all over the world. In susceptible apple, pear and quince varieties, ASGV is associated with economically important diseases such as apple stem pitting, apple epinasty and -decline, pear red mottle, pear stony pit and quince sooty ring spot as well as fruit deformations. Other symptoms include chlorosis, leaf distortion and/or a non-specific gloss to the cultivar (Desvignes *et al*., 1999; Leone *et al*., 1998; Maxim *et al*., 2004).

The ASGV genome is a positive sense, single-stranded, polyadenylated RNA genome and between 6495 and 6497 nucleotides in length (De Sequeira and Lister, 1969; Yoshikawa *et al*., 1992; Tatineni *et al*., 2009), excluding a 3'-terminal poly(A) tail (Yoshikawa *et al*., 1992). The CP gene variability among *Capillovirus* isolates from distinct regions is very low (Nickel *et al*., 2001). The CP is the most abundantly expressed viral protein since large quantities are required for virion formation (Callaway *et al*., 2001). The CP of Indian ASGV isolate from the apple variety Starkrimson share 100% identity with the Brazilian isolate AF438409 (Negi *et al*., 2010). ASGV is very closely related to *Potato Virus T* (PVT), although their genome organizations are quite different (Yoshikawa and Takahashi, 1988). ASGV also shows striking genome similarities with CTLV. Both CTLV and PVT belong to the genus *Capillovirus*. Very high amino acid identities of 95-100% have also been found between the coat proteins of ASGV and CTLV, indicating CTLV to be a very close relative of ASGV within the genus *Capillovirus* (Yoshikawa *et al*., 1993; Negi *et al*., 2009).

In this study, the occurrence levels of ASGV infection in pome fruit trees in South Africa was assessed using RT-PCR. Furthermore, the genetic variation of random selected ASGV isolates found in South Africa was assessed by RT-PCR amplification of their coat protein sequences and sequencing. The generated sequences were aligned and phylogenetically analyzed with ASGV CP sequences obtained from GenBank. Phylogenetic analyses of CP gene sequence matrices and matrices, in which CP gene sequences and whole genome

sequences were combined in a supermatrix, were performed with the objective of accurately determining the phylogenetic relationships of the South African isolates.

4.2. Materials and methods

4.2.1. Sampling

A total of 65 leaf samples that tested positive for the presence of ASGV with the DAS-ELISA during three of SAPO Trust laboratory's active testing seasons (between September 2011 and December 2013) were tested for the presence of ASGV using RT-PCR. Samples included nucleus greenhouse material, foundation block material as well as mother block material. Samples were stored at 4^{o}C and prepared for RT-PCR within a period of five days after the ELISA tests were done.

4.2.2. Sample preparation

Samples were prepared by stacking ten leaves onto each other and by using sterile scalpel, a representative sample (leaf tissue from each leaf in the sample) of 0.1 g was cut. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany). RNA was eluted to a final volume of 40 µl.

4.2.3. RT-PCR for the amplification of the ASGV CP gene of isolates

The PCR mixture was prepared containing 2.5 µl 10x PCR buffer (Bioline, UK), 1.25 µl of 0.1 M DTT, 1 µl ASGV forward primer (20 µM), 1 µl ASGV reverse primer (20 µM), 1 µl of 5 mM dNTP's (Bioline, UK), 0.25 µl of 5 U/µl Taq^{TM} DNA polymerase (Bioline, UK), 0.125 µl of 200 U/µl SuperScriptTM III reverse transcriptase (Invitrogen, USA). A total volume of 23 µl sample mixture was used together with 2 µl of sample RNA and 14.875 µl Milli-Q$^{®}$ water to have a total reaction volume of 23 µl. Primers used are listed in Table 4.1. A total of 2 µl of isolated plant RNA was added to 23 µl RT-PCR reaction mixtures. The one-step RT-PCR was performed by reverse transcriptase at 48°C for 30 minutes followed by 40 cycles of the following parameters: denaturation at 96°C for 30 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 60 seconds with a final elongation at 72°C for 10 minutes. Amplifications were performed using a Applied Biosystems Veriti Thermocycler (Thermo Fisher Scientific, US). The primers used for ASGV RT-PCR and sequencing as well as the size of the ASGV CP gene amplicon are listed in Table 4.1.

Table 4.1: Primer sequences and expected size for ASGV detection and sequencing used in this study.

Primer*	**Primer sequence in 5'-3' orientation**	**Product size**
Forward	GTCCCTCTCGGCTAGAATTGAAAGAT	780 bp
Reverse	GCGACCAAGTTTGCGGAATTTCACA	

Primers were designed by Dr. C. Visser, Department of Biochemistry, University of Stellenbosch, South Africa.

4.2.4. Agarose gel electrophoresis

RT-PCR product formation was confirmed using agarose gel electrophoresis. A 1% (w/v) agarose gel was prepared with 1 × TAE buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and Milli-Q® water. Ethidium bromide (Sigma) was added to visualize products under UV light. The electrophoresis buffer consisted of 1 × TAE buffer. Ten microliters of the PCR reaction mixture were mixed with loading buffer (Bioline, UK) and loaded on the agarose gel. Electrophoresis was performed at 100 V for 80-100 minutes using a power source (Cleaver Scientific, UK). A 100 bp DNA size marker ladder (Bioline, UK) was used to determine the size of amplified DNA fragments. The DNA bands were visualized under a UV transilluminator and portable darkroom (Cleaver Scientific, UK). Amplification was regarded as successful where visual bands of the anticipated fragment length were observed.

4.2.5. RT-PCR product purification

In order to confirm the identity of the RT-PCR products, amplicons were purified and sequenced. These products were purified using a Wizard SV Gel and PCR Clean-up System (Promega, US) according to the manufacturer's instructions. The final purified PCR products were eluted in 40 µl of Milli-Q® water. Purified products were separated by gel as described above to assess DNA yield and were stored in nuclease-free Eppendorf tubes at -20°C until sequencing.

4.2.6. Dye terminator sequencing reaction

The RT-PCR products were sequenced with the Cycle Sequencing (Applied Biosystems, California, US). The sequencing reaction mixture consisted of 5 µl of 5 × sequence dilution buffer (Applied Biosystems, California, US), 2 µl Terminator Dye (Big Dye® Terminator v3 Cycle Sequencing kit, Applied Biosystems, California, US), 2 µl of the cDNA template

solution and 1 µl primer (0.8 µM). The same combinations of forward and reverse primers used for amplification were also used for sequencing reactions (Table 4.1). The thermal cycling program consisted of 35 cycles of 96°C for 10 s, 52°C for 30 s, 60°C for 4 min followed by an elongation step at 60°C for 7 min. The cycle sequencing products were analyzed using ABI®3730xl Genetic Analyser in the Central Analytical Facility, University of Stellenbosch.

4.2.7. Sequence analysis and alignment

The sequence chromatograms resulting from the forward and reverse dye terminator sequencing reactions from South African ASGV isolates were edited using ChromasPro version 1.5 (Technelysium Pty., Ltd.) and combined to produce consensus sequences. The generated sequences were identified as ASGV CP genes using the basic local alignment search tool (BLAST) on GenBank. The generated sequences were aligned with ASGV and CTLV CP gene sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov) (GenBank) (http://blast.ncbi.nlm.gov/Blast.cgi) (listed in Table 4.2) using BioEdit version 7.0.5.2 software package (Hall, 1999). CP gene sequences of *Cherry virus A* (CVA) (EU862278 and EU862279) were used as outgroups. Gaps were treated as unknown characters and replaced with question mark characters.

In an attempt to obtain greater resolution in the phylogenetic trees additional analyses were performed. Firstly, ASGV and CTLV whole genome sequences were downloaded from GenBank (listed in Table 4.3), aligned as above, and subjected to phylogenetic analysis. Subsequently, ASGV and CTLV CP gene sequences were also aligned with ASGV and CTLV whole genome sequences, and subjected to phylogenetic analysis.

Table 4.2 Details of ASGV CP gene isolates obtained from GenBank used for phylogenetic analysis.

GenBank Accession number	**Virus**	**Host**	**Country of origin**	**Reference**
EU862278	CVA	Cherry	China	Rao *et al*., 2009.
EU862279	CVA	Cherry	China	Rao *et al*., 2009.
AY596172	PBNLS	Pear	South Korea	Yang and Min (unpublished)
AB004063	ASGV	Lily	Japan	Terauchi *et al*., 1997
AF438409	ASGV	Apple	Brazil	Nickel *et al*., 2001
AF465354	ASGV	Pear	Korea	Shim and Lee (unpublished)
AY886760	ASGV	Pear	China	Guo *et al*., 2005
EU236258	ASGV	Apple	China	Yue and Wu (unpublished)
FJ608985	ASGV	Pear	China	Zheng *et al*., 2006
GQ330293	ASGV	Pear	China	Zheng *et al*., 2006
GQ330294	ASGV	Pear	China	Zhen *et al*., 2006
JN792487	ASGV	Pear	South Korea	Kim *et al*. (unpublished)
JX885576	ASGV	Apple	China	Liu *et al*., 2013
JX885571	ASGV	Apple	China	Liu *et al*., 2013
JN871589	ASGV	Apple	Yunnan	Ji *et al*. (unpublished)
FM204881	ASGV	Apple	India	Negi *et al*. (unpublished)
D14455	CTLV	Unknown	Unknown	Ohira *et al*., 1994
JN792475	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792481	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792483	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792473	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792476	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792474	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JN792477	ASGV	Apple	South Korea	Kim *et al*. (unpublished)
JX885580	ASGV	Apple	China	Liu *et al*., 2013
FJ952166	ASGV	Apple	Turkey	Gadiou *et al*., 2010
FJ952159	ASGV	Apple	India	Gadiou *et al*., 2010
FJ952160	ASGV	Apple	India	Gadiou *et al*., 2010
FJ952164	ASGV	Apple	India	Gadiou *et al*., 2010
FJ952165	ASGV	Apple	India	Gadiou *et al*., 2010
FJ952161	ASGV	Apple	Czech Republic	Gadiou *et al*., 2010
FJ952162	ASGV	Apple	Czech Republic	Gadiou *et al*., 2010
FJ952163	ASGV	Apple	Serbia	Gadiou *et al*., 2010

Table 4.3 Details of ASGV complete genome sequence isolates obtained from GenBank used for phylogenetic analysis.

GenBank Accession number	Virus	Host	Country of origin	Reference
EU862278	CVA	Cherry	China	Rao *et al*., 2009.
EU862279	CVA	Cherry	China	Rao *et al*., 2009.
AY646511	CTLV	Kumquat	Taiwan	Lin *et al*. (unpublished)
D14995	ASGV	Apple	Japan	Yoshikawa *et al*., 1992
D16368	CTLV	Unknown	Japan	Yoshikawa *et al*., 1993
FJ355920	CTLV	Naval orange	Taiwan	Lin *et al*. (unpublished)
NC001749	ASGV	Apple	Japan	Yoshikawa *et al*., 1992
JQ308181	ASGV	Apple	China	Zhao *et al*., 2012
JN701424	ASGV	Pear	China	Hu *et al*. (direct submission)
JQ765412	CTLV	Citrus	China	Bu and Ding (direct submission)
JX080201	ASGV	Apple	Germany	Liebenberg *et al*., 2012
JX416228	CTLV	Orange	Taiwan	Direct submission
KC588947	CTLV	Orange	China	Song *et al*. (unpublished)
KC588948	CTLV	Orange	China	Song *et al*. (unpublished)
HE978837	ASGV	Apple	India	Dhir *et al*. (unpublished)

4.2.8. Phylogenetic analysis

Phylogenetic analyses was performed with the software program “Phylogenetic analysis using parsimony (PAUP) version 4.0b10” (Swofford, 2002). The CP gene sequences of 30 ASGV and CTLV isolates obtained from GenBank (Table 4.2), CP gene sequences generated in this study from South Africa isolates and the whole genome sequences of 13 ASGV and CTLV isolates (Table 4.3) obtained from GenBank were used in phylogenetic analyses using parsimony. A heuristic search was used to establish the shortest possible trees from the sequence alignment data matrices. The search criteria included the use of 1000 addition sequence replicates with the tree bisection and reconstruction (TBR) branch swapping algorithm. Gaps were treated as missing data. All characters were unordered and equally weighted. Bootstrap support values were calculated from the 1000 heuristic search replicates and 10 random taxon additions to determine clade support. Bootstrap values less than 50% were considered weakly supported and were not indicated on phylograms. Values from 50% to 74% were considered moderately supported and were indicated on phylograms. Branches with bootstrap values of 75% and higher were considered as well supported. Parsimony included consistency index (CI) and retention index (RI) values (Farris, 1994).

A total of three phylogenetic analyses were performed. In the first analysis the ASGV CP gene sequences (Table 4.2), CP gene region from the 13 complete ASGV and CTLV whole genome sequences (Table 4.3) as well as the CP gene sequences of South African ASGV isolates were included for phylogenetic analysis. In the second analysis the 13 complete ASGV and CTLV whole genome sequences (Table 4.3) were included in the phylogenetic analysis. In the third analysis the CP gene sequences of South African ASGV isolates, ASGV CP gene sequences (Table 4.2) as well as complete ASGV and CTLV whole genome sequences (Table 4.3) of isolates obtained from GenBank were included. The gaps from the much longer ASGV genome sequences were treated as question marks in the analyses. Sequences of isolates EU862279 (CVA) and EU862278 (CVA) were used as outgroups for all three analyses.

4.3. Results

4.3.1 DAS-ELISA and electrophoretic analysis of amplified products of ASGV using RT-PCR

Figure 4.1 shows the RT-PCR products separated by gel electrophoresis of a representative seven of the 64 samples. The RT-PCR amplicons of all 64 samples were of the expected size of approximately 780 bp. The virus status of all 64 samples that tested positive for the presence of ASGV with DAS-ELISA could be confirmed with RT-PCR. Samples were collected from all over South Africa's pome fruit producing areas (Table 4.4).

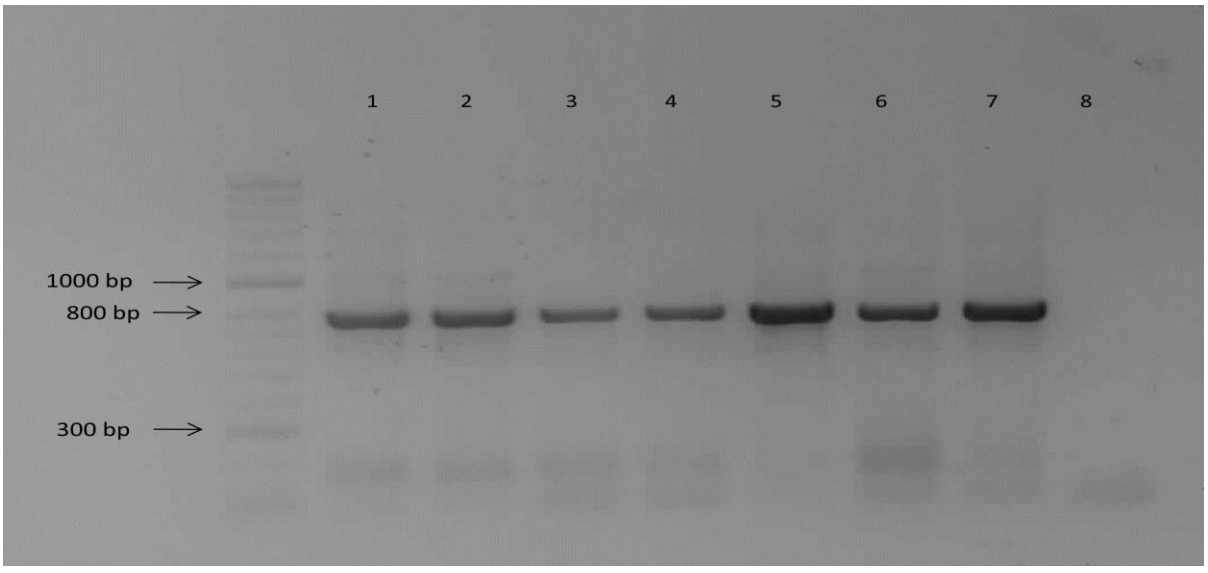

Figure 4.1. RT-PCR results of seven samples of the ASGV CP gene sequences. RT-PCR products were of the expected size of 780 bp. Lane 1: Bioline 100 bp Hyperladder^{TM} II DNA markers. Lane 2: Sample MC1. Lane 3: Sample MC5. Lane 4: Sample MC11. Lane 5: Sample MC15. Lane 6: Sample 26. Lane 7: Sample MC35. Lane 8: Sample MC46. Lane 9: Negative control.

Table 4.4. The list of pome fruit samples used in this study. Samples in which virus detection and/or sequence analysis was successful are marked ■ Samples in which virus detection and/or sequence analysis was unsuccessful are marked with □.

Collection number	Fruit type	Sample Region	ASGV		
			DAS-ELISA	RT-PCR	CP gene sequencing RT-PCR
			■	■	■
MC2	Apple	Stellenbosch	■	■	■
MC3	Apple	Stellenbosch	■	■	■
MC5	Apple	Stellenbosch	■	■	■
MC7	Apple	Stellenbosch	■	■	■
MC8	Apple	Stellenbosch	■	■	■
MC9	Apple	Stellenbosch	■	■	■
MC10	Apple	Stellenbosch	■	■	■
MC11	Apple	Stellenbosch	■	■	■
MC12	Apple	Stellenbosch	■	■	■
MC13	Apple	Ceres	■	■	■
MC14	Apple	Ceres	■	■	■
MC15	Apple	Villiersdorp	■	■	■
MC16	Apple	Villiersdorp	■	■	■
MC17	Apple	Ceres	■	■	■
MC18	Apple	Grabouw	■	■	■
MC19	Apple	Grabouw	■	■	□
MC20	Apple	Unknown	■	■	■
MC21	Apple	Unknown	■	■	■
MC22	Apple	Unknown	■	■	■
MC23	Apple	Unknown	■	■	■
MC24	Apple	Unknown	■	■	■
MC25	Apple	Stellenbosch	■	■	■
MC26	Apple	Stellenbosch	■	■	■
MC27	Apple	Stellenbosch	■	■	■
MC28	Apple	Stellenbosch	■	■	□
MC29	Apple	Stellenbosch	■	■	■
MC30	Apple	Unknown	■	■	■
MC31	Apple	Unknown	■	■	■
MC32	Apple	Unknown	■	■	■
MC33	Apple	Unknown	■	■	■
MC34	Apple	Unknown	■	■	■
MC35	Apple	Unknown	■	■	■
MC36	Apple	Stellenbosch	■	■	■
MC37	Apple	Stellenbosch	■	■	■
MC38	Apple	Stellenbosch	■	■	■
MC39	Apple	Stellenbosch	■	■	□
MC40	Apple	Stellenbosch	■	■	□
MC41	Apple	Unknown	■	■	□
MC43	Apple	Unknown	■	■	□
MC44	Apple	Stellenbosch	■	■	■

Collection number	Fruit type	Sample Region	ASGV		
			DAS-ELISA	RT-PCR	CP gene sequencing RT-PCR
MC45	Apple	Unknown	■	■	□
MC46	Apple	Unknown	■	■	■
MC47	Apple	Stellenbosch	■	■	■
MC48.1	Apple	Stellenbosch	■	■	■
MC48.2	Apple	Stellenbosch	■	■	■
MC49.1	Apple	Stellenbosch	■	■	■
MC49.2	Apple	Stellenbosch	■	■	■
MC50	Apple	Stellenbosch	■	■	■
MC51	Apple	Stellenbosch	■	■	■
MC52	Apple	Stellenbosch	■	■	■
MC53	Apple	Stellenbosch	■	■	■
MC54	Apple	Stellenbosch	■	■	□
MC55	Apple	Stellenbosch	■	■	■
MC56	Apple	Stellenbosch	■	■	■
MC57	Apple	Stellenbosch	■	■	■
MC59	Apple	Grabouw	■	■	■
MC60	Apple	Grabouw	■	■	■
MC61	Apple	Ceres	■	■	■
MC62	Apple	Grabouw	■	■	■
MC63	Apple	Grabouw	■	■	■
MC64	Apple	Grabouw	■	■	■
MC65	Apple	Riviersonderend	■	■	■
MC66	Apple	Riviersonderend	■	■	■

4.3.2. Sequence analysis of ASGV amplified products

RT-PCR products of 56 of the 64 ASGV infected samples that were successfully separated by agarose gel electrophoresis by showing bands of the expected size were successfully sequenced (Table 4.4).

4.3.3. Phylogenetic analysis of ASGV CP gene- and ASGV complete genome sequences using parsimony

In the phylogenetic analysis performed on ASGV and CTLV CP gene sequences and CP gene regions within ASGV whole genome sequences using parsimony, the heuristic search retrieved a total of 80 trees with a tree length of 1200. The analysis revealed that a total of 249 (31.6%) characters were constant, 55 (7.0%) characters were parsimony uninformative and 484 (61.4%) characters were parsimony informative. The tree statistics revealed a CI of 0.590 and a RI of 0.854. A single tree is shown in Figure 4.2. A strict consensus tree is shown in Figure 4.3. Most isolates grouped in strongly supported clades, but although the relationships between these clades were resolved, they were not supported. Thus the closest relatives of two of the groups of South African isolates (Group 1 consisting of isolates

MC47 and MC48.1, group 2 consisting of isolates (MC1, MC3, MC5, MC12, MC17, MC21, MC25, MC38, MC46, MC48.2, MC51-53, MC56 and MC65) could not be established. However, a third group (MC2, MC7-11, MC13-16, MC18, MC20, MC22, MC26, MC27, MC30-36, MC44, MC49.1, MC49.2, MC50, MC55, MC57, MC 59-64 and MC66) grouped in a strongly supported clade with isolates from Brazil, Europe and Asia.

The CP gene sequences of isolates MC3, MC5, MC21 and MC46 from group 2 have unique sequences between nucleotide position 5716 and 5726 in the alignment that are completely different from all other South African group 2 isolates. This is reflected by the long branch length of this group of isolates and could be seen to be a subgroup of group 2. The short branch lengths between these isolates show that little variation exists amongst them (96.2 to 97.8% identity, calculated in relation to the length of the CP gene sequence alignment).

In the phylogenetic analyses performed on 13 complete ASGV and CTLV whole genome sequences using parsimony, the heuristic search retrieved one tree with a tree length of 4488. The analysis revealed that a total of 3984 (61.2%) characters were constant, 535 (8.2%) characters were parsimony uninformative and 1993 (30.6%) characters were parsimony informative. The tree statistics revealed a CI of 0.691 and a RI of 0.743. The single tree is shown in Figure 4.4. The tree was resolved and nodes were retrieved with high bootstrap support. This tree therefore shows how these isolates are related to one another.

In the phylogenetic analysis that was performed on ASGV and CTLV CP gene sequences and whole genome sequences using parsimony, the heuristic search retrieved a total of 800 trees with a tree length of 1970. The analysis revealed that a total of 877 (48.5%) characters were constant, 100 (5.5%) characters were parsimony uninformative and 833 (46.0%) characters were parsimony informative. The tree statistics revealed a CI of 0.649 and a RI of 0.8796. A single tree is shown in Figure 4.5. A strict consensus tree is shown in Figure 4.6. The strict consensus tree was partially resolved and a number of the major clades were retrieved with high bootstrap support. In comparison to the phylogeny computed with CP gene sequences only (Figure 4.2 and 4.3), the South African isolates in group 2 were now retrieved in a clade with Japanese and South Korean isolates. This shows that the South African isolates in group 2 are related to Japanese and South Korean isolates. However, the South African isolates in group 1 were retrieved in an unresolved position in the phylogeny (see Figure 4.5), sister to other Japanese and South Korean isolates, but in the strict consensus tree (Figure 4.6) this group becomes a clade in the basal polytomy. The third group (MC2, MC7-11, MC13-16, MC18, MC20, MC22, MC26, MC30-36, MC44, MC49.1, MC49.2, MC50, MC55, MC57, MC 59-64 and MC66) was again retrieved in a strongly supported clade with isolates from Brazil, Europe and Asia.

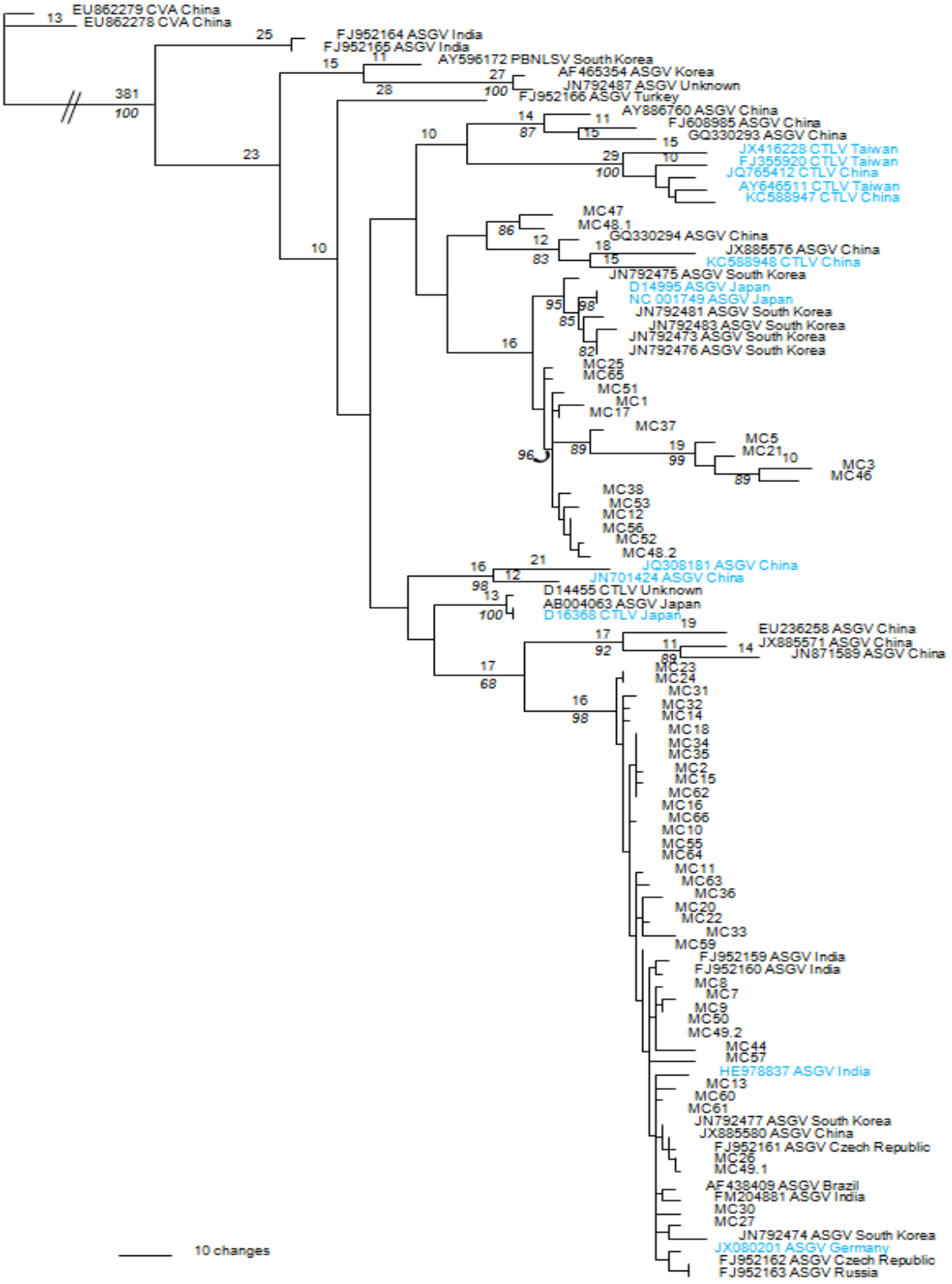

Figure 4.2. One of the shortest trees retrieved from a heuristic search performed on the ASGV CP gene sequence data matrix of South African isolates as well as the CP gene sequences and CP gene regions of international ASGV and CTLV whole genome isolates. Branch lengths ≥10 are indicated above branches and bootstrap values below branches in italics. The branch length of the CVA outgroups was trimmed to fit this page. South African isolates are indicated with the lettering starting with MC. Isolates for which genome sequences are available on GenBank are indicated in blue.

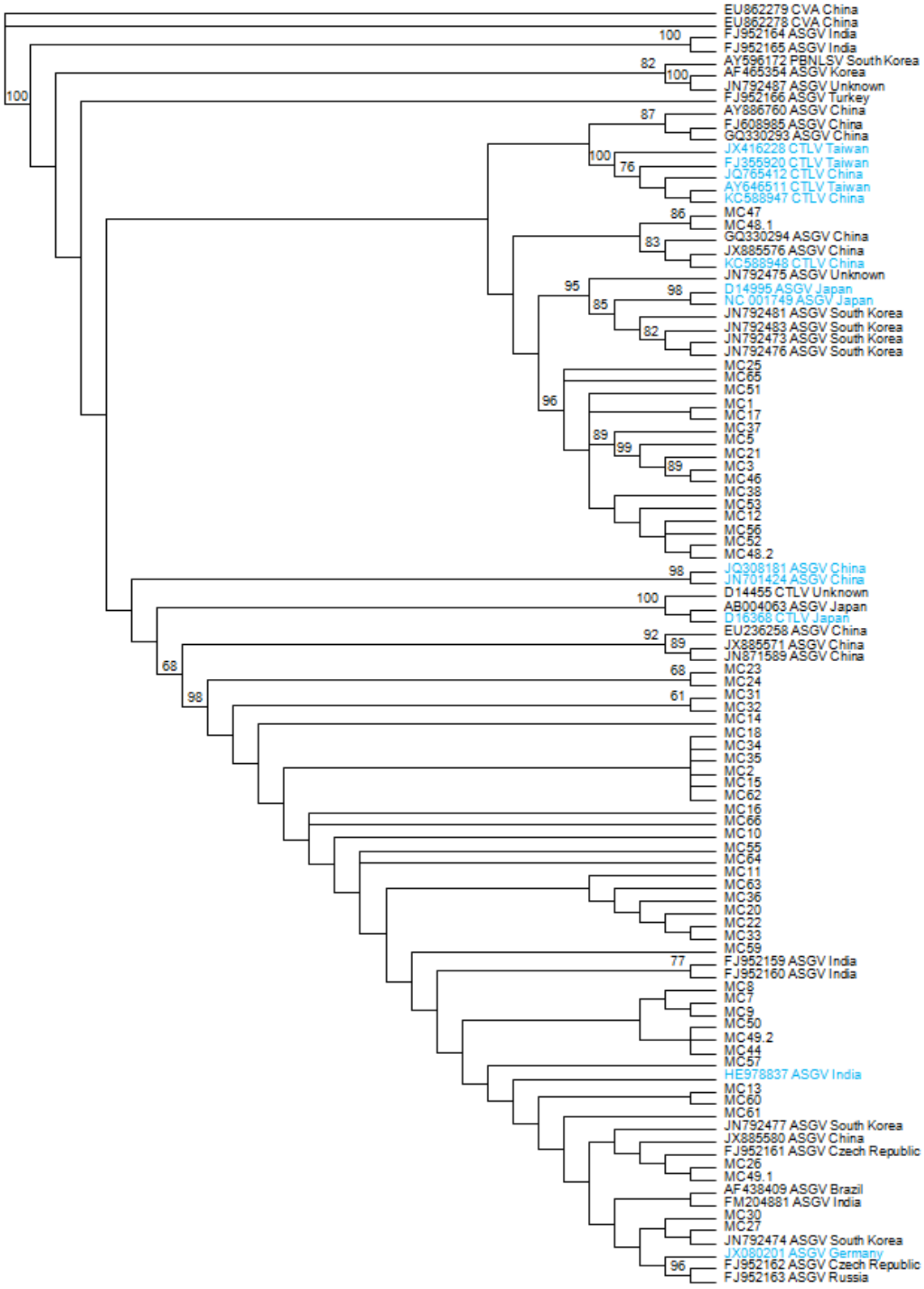

Figure 4.3. The strict consensus of one the shortest trees retrieved from a heuristic search performed on the ASGV CP gene sequence data matrix of South African isolates as well as the CP gene sequences and CP gene regions of ASGV and CTLV whole genome isolates of international isolates. Bootstrap values are indicated above branches. South African isolates are indicated with the lettering starting with MC. Complete genome sequences are indicated in blue. CVA (isolate EU862279) was used as the outgroup.

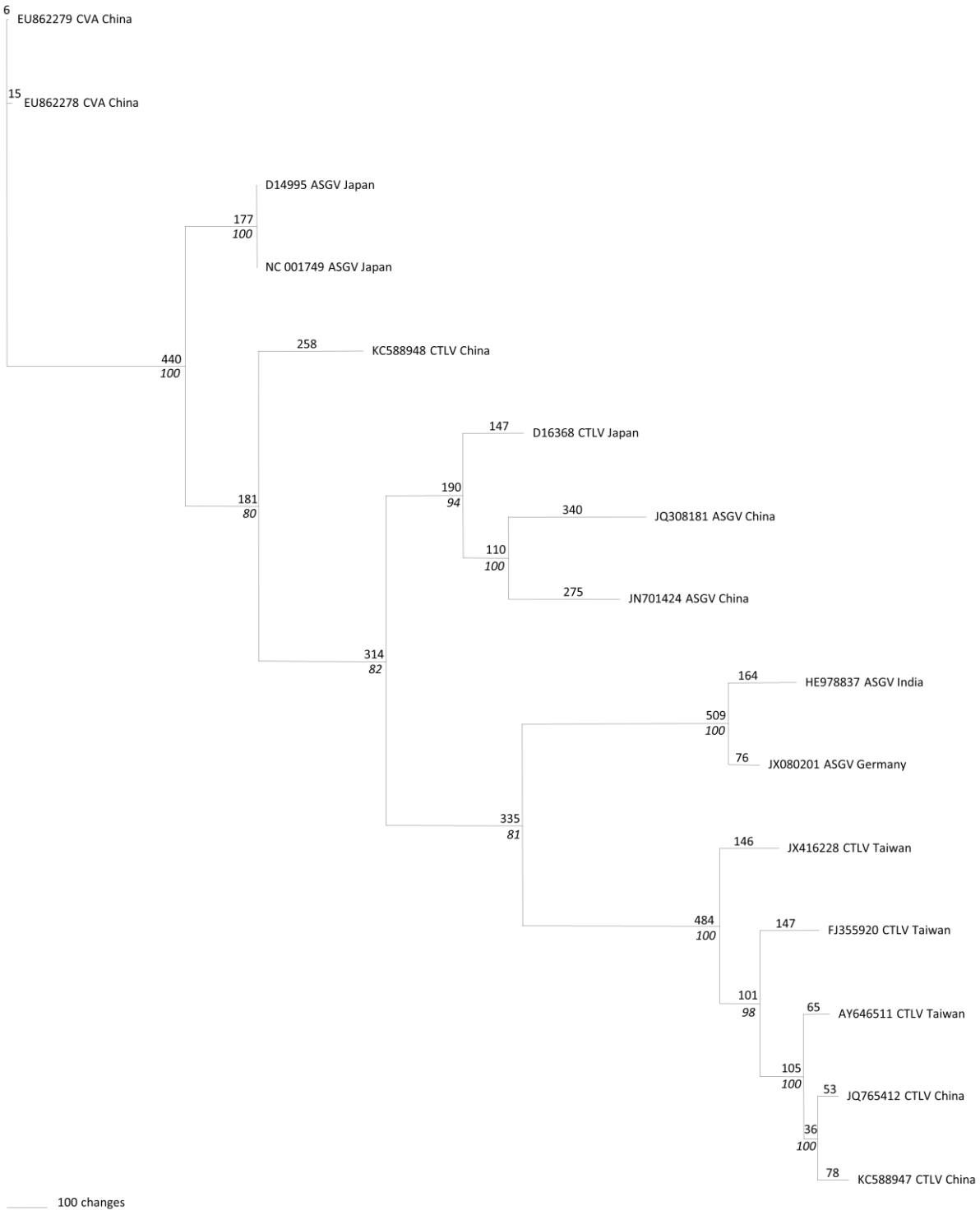

Figure 4.4. The shortest tree of a heuristic search performed on the ASGV and CTLV complete genome sequence data matrix. Branch lengths are indicated above branches. Bootstrap values are indicated underneath branches in italics. CVA (isolate EU862279) was used as the outgroup.

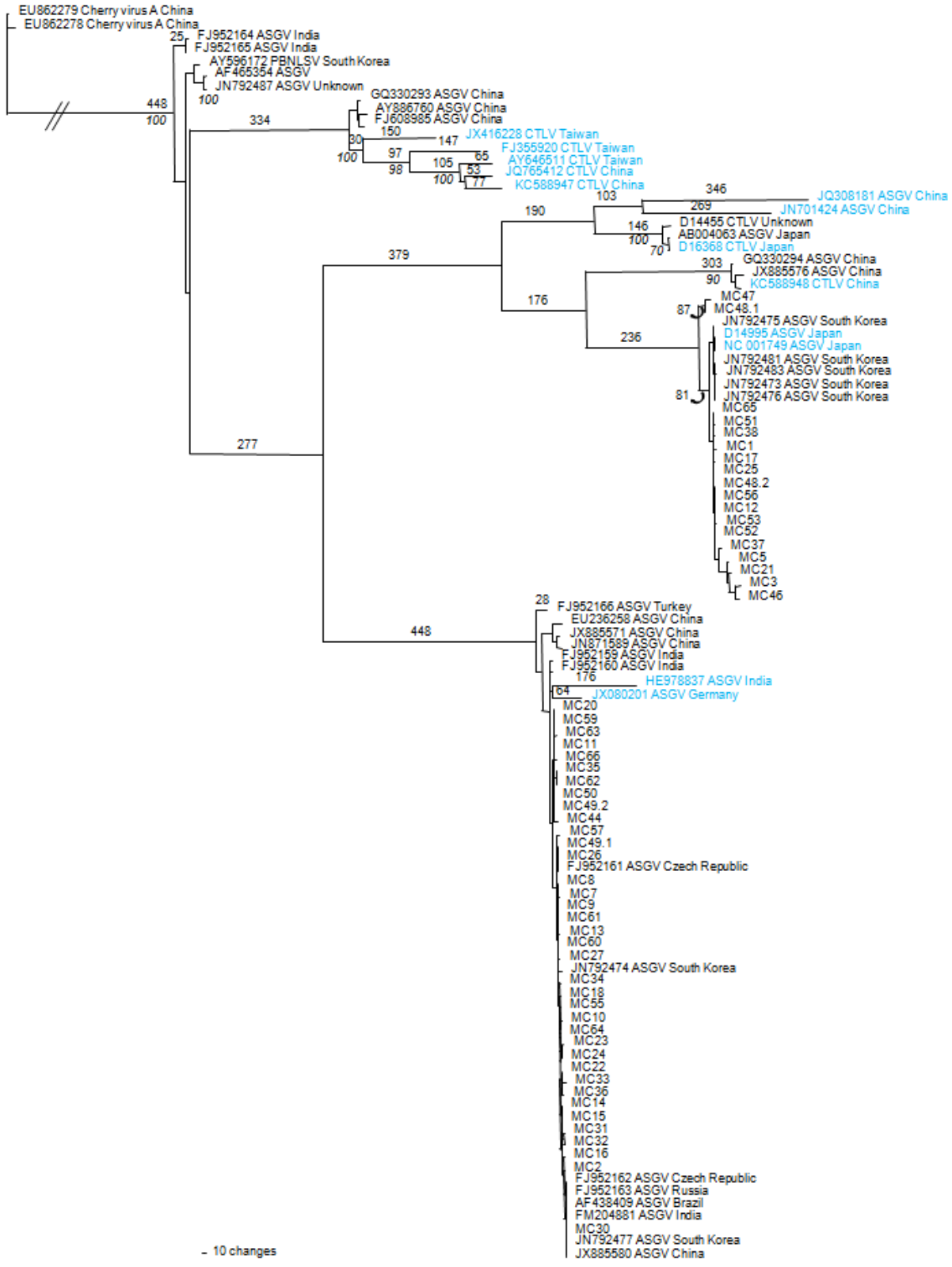

Figure 4.5. One of the shortest trees of a heuristic search performed on the ASGV and CTLV CP gene and complete genome sequence data matrix. Branch lengths ≥25 are indicated above branches and bootstrap values below branches in italics. The branch length of the CVA outgroups was trimmed to fit this page. South African isolates are indicated with the lettering starting with MC. Isolates for which genome sequences are available on GenBank are indicated in blue.

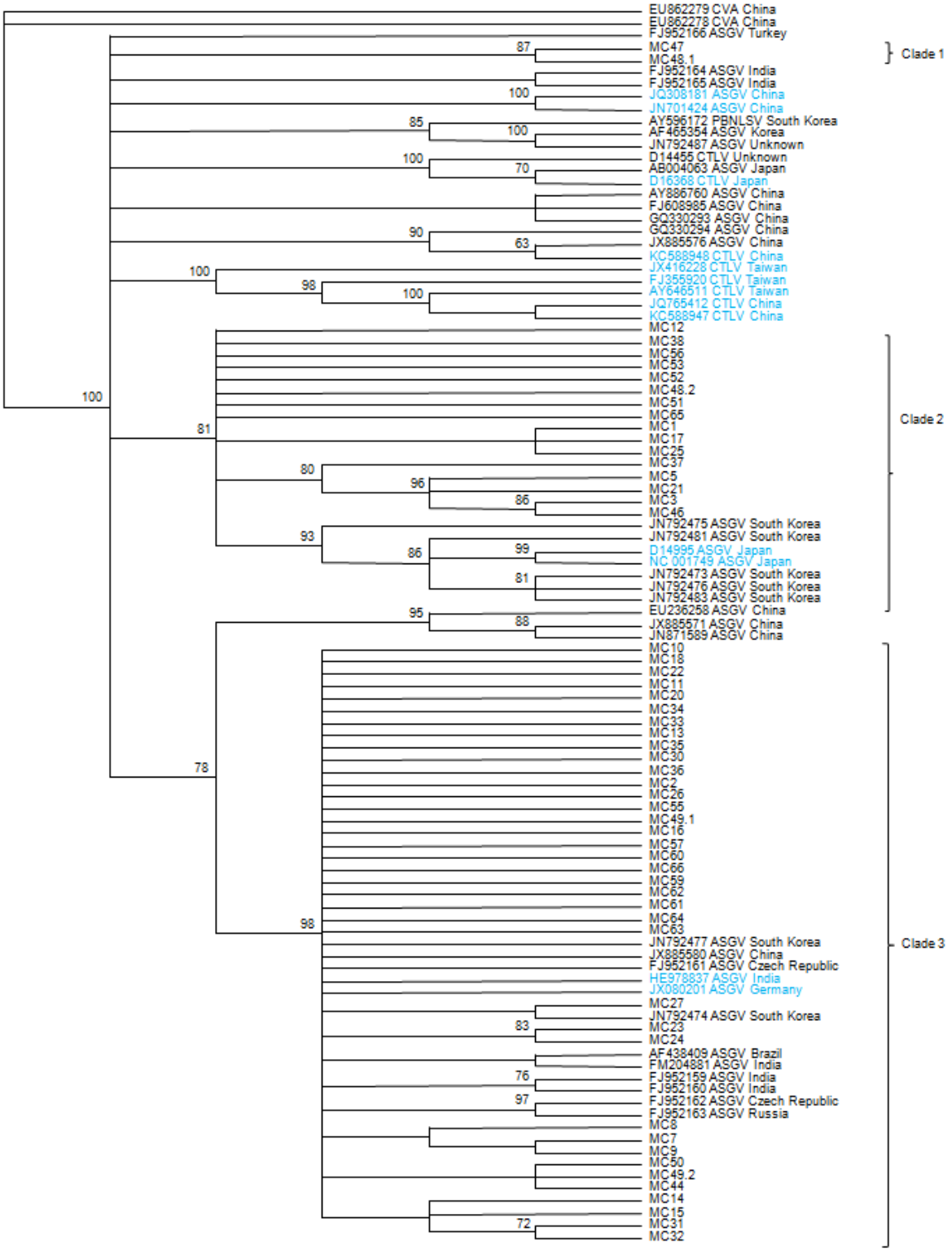

Figure 4.6. The strict consensus of one the shortest trees retrieved from a heuristic search performed on the ASGV and CTLV CP gene sequence and complete genomes data matrix. Bootstrap values are indicated above branches. South African isolates are indicated with the lettering starting with MC. Isolates for which genome sequences have been generated are indicated in blue. CVA (isolate EU862279) was used as the outgroup.

4.4. Discussion

In the first phylogenetic analysis that was performed on the South African ASGV CP gene sequences and ASGV and CTLV CP gene- and CP gene regions of ASGV and CTLV whole genome sequences obtained from GenBank, a phylogenetic tree was retrieved in which the basal nodes showed low bootstrap support. Although three groups of South African isolates could be identified, only the nearest relatives of group 3 could be determined, i.e. a collection of isolates from Brazil, Europe and Asia.

In an attempt to obtain better resolution, a second phylogenetic analysis was performed on ASGV and CTLV whole genome sequences obtained from GenBank only. This analysis produced a completely resolved and well supported tree. This indicates that sufficient variation exists within these whole genome sequences to resolve the basal nodes in the phylogeny.

Following the information gathered from the second analysis, a third analysis was performed which included South African ASGV CP gene sequences, and ASGV and CTLV CP gene- and whole genome sequence data from other parts of the world. The procedure of incorporating partial sequences in matrices of larger full length sequences in order to obtain the phylogenetic position of the taxon possessing the shorter sequence is referred to as the "supermatrix approach" in phylogenetic analysis (De Queiroz and Gatesy, 2006; Linder, 2008; Pirie *et al*., 2008). The tree resulting from this analysis shows an improvement on the tree established from CP gene sequences only in that the relationships of the isolates in group 2 can be reduced i.e. their closest relatives are isolates from Japan and South Korea. However, the clade consisting of group 1 is still retrieved in an unresolved position and its relationships are unclear. From the short branch lengths in the clades in which groups of South African isolates (group 1, 2 and 3) are retrieved in the third analysis, it can be seen that little variation exists amongst the different isolates in each of the respective groups.

The phylogenetic analysis furthermore indicates the possible origins of South African isolates. The origin of group 2 isolates are in all likelihood Europe or Asia. The single isolate from Brazil is also likely to have been imported from Europe or Asia as the bulk of the overseas isolates are from those areas. The isolates in group 3 clearly originate from Japan or South Korea i.e. from a relatively small geographic area. The origins of group 1 could not be established because of the unresolved position in the phylogeny. Thus the geographic origins of two of the three groups of South African isolates could be determined with a reasonable degree of certainty. This indicates that South African ASGV isolates are likely to have been imported with infected material from overseas and not from local origins.

In this study a South Korean PBNLS disease sequence (AY596172) was included for phylogenetic analysis. It showed a close relationship with ASGV CP gene sequences AF465354 (Korea) and JN792487 of unknown origin. In 2004, it was reported that PBNLS disease could be caused by ASGV (Shim *et al*., 2004) and this phylogeny supports this deduction. In 2006, a further study by Shim *et al*. examined the pathogenicity of the ASGV Korean isolate ASGV-K in pear trees and other experimental hosts when carried by the fungal vector *Talaromyces flavus*. It was found that ASGV-harboring *T. flavus* induced mild PBNLS symptoms on virus-free pears indicating that *T. flavus* may be a vector of ASGV.

As shown in the whole genome sequence phylogeny, CTLV isolates group within a monophyletic ASGV clade. Research has shown that Apple stem grooving virus shows striking genome similarities with CTLV which is therefore confirmed in this study. A study performed by Yoshikawa *et al*. (1993) showed that the CTLV genome sequence of the 3'-terminal (excluding the poly(A) tail) shows 86.1% identity to that of the ASGV genome. Similarities of amino acid sequences encoded by ORF1 and ORF2 of CTLV with the corresponding regions of ASGV are 86.1% and 97.3% respectively. Very high amino acid identities of 95-100% have also been found between ASGV and CTLV, indicating CTLV to be a very close relative of ASGV within the genus *Capillovirus* (Negi *et al*., 2009).

Although three groups of South African ASGV isolates with a possible subgroup in group 2 were identified, the pathogenicity of these groups was not investigated. In the context of the threats that these groups of isolates may hold for the South African pome fruit industry it would be important to establish whether their symptom expression and pathogenicity is different. A project that will investigate the pathogenicity of South African isolates may be an objective for future research.

Chapter 5. The detection and determination of the genetic variation of Apple stem pitting virus in South African orchards based on CP gene sequences

5.1. Introduction

Apple stem pitting virus is a latent pome fruit virus infecting apple and pear trees worldwide. The virus is mainly distributed in the palisade tissue of mesophyll cells, external cortex of the shoot tip, and the newly formed vascular bundles (Zhao *et al*., 2009). ASPV is a flexuous, filamentous virus that is 12-15 nm wide and 800 nm long and is found in the cytoplasm of mesophyll cells. The virus is comprised of a single stranded positive-sense RNA with a M_r of 3.1 x 10^6 and a major CP of M_r 48 000 (Koganezawa and Yanase, 1990). The complete genomes of different ASPV isolates that have been sequenced vary in length between 9265 and 9310 nucleotides (Jelkman, 1994; Yoshikawa *et al*., 2001; Liu *et al*., 2012). Komorovska *et al*. (2011) compared a total of ten apple and pear ASPV isolates which revealed high diversity of the CP gene as well as one or two deletions in the N-terminal part of the CP gene. A study performed by Gadiou *et al*. (2010) found the genetic variability of the ASPV CP gene region at nucleotide level to be between 80.1% and 81.9%.

In many countries, plants used for propagation material and plants grown as part of a fruit production program are regularly inspected visually to diagnose viral diseases. Since latent viruses are by definition viruses that do not show any visual symptoms on commercial fruit varieties, visual inspection as a method of viral detection is inadequate. Many cultivated fruit crops are maintained through vegetative propagation which makes the detection of viruses complicated since mixed infection with several viruses is a common phenomenon. ASPV can be spread through mechanical transmission by means of infected pruning equipment. To date, there is no known natural vector for ASPV. ASPV often occurs in a complex with other latent pome fruit viruses such as ASGV, ACLSV and ApMV (Caglayan *et al*., 2006; Kundu, 2003; Nickel *et al*., 2001). Previous studies done by Kundu (2003) and Caglayan *et al*. (2006) yielded an ASPV and ASGV mixed infection rate of 36.8 and 14.7% respectively.

In susceptible apple, pear and quince varieties, ASPV is associated with economically important diseases such as apple stem pitting, apple epinasty and -decline, pear red mottle, pear stony pit, quince sooty ring spot and fruit deformations (Leone *et al*., 1998; Desvignes *et al*., 1999). ASPV has also been reported to cause PVYV in pear (Jelkmann, 1994; Leone *et al*., 1998; Nemchinov *et al*., 1998; Rossini *et al*., 2010). Paunovic and Rankovic (1998) found evidence that allows a preliminary identification of quince fruit deformation virus as a strain of PVYV and/or ASPV. Paunovic *et al*. (1999) found that a sample from the pear cultivar Württemberg associated with pear stony pit was an isolate of ASPV, but it is not

known yet whether ASPV is the causal agent for pear stony pit disease. However, pear stony pit disease has been suspected to be caused by ASPV (Jelkman, 1994; Leone *et al*., 1995; Leone *et al*., 1998; Paunovic and Rankovic., 1998; Desvignes *et al*., 1999; Paunovic *et al*., 1999; Brakta *et al*., 2013). During 2014, the pear stony pit syndrome has been reported to also occur in certain pear cultivars in South Africa (F.G.H. Van Zyl, personal communication).

In South Africa, the only method currently described by the South African Deciduous Fruit Certification Scheme for the determination and identification of ASPV in pome fruit trees for certification purposes is biological indexing. Biological indexing for ASPV is a field testing method which can take up to three years. This method of biological indexing by grafting onto the woody indicator Virginia Crab and Spy 227 can be described as a 'baseline' test for the certification of propagation material. Apple stem pitting virus is known to cause xylem pits in the stem of the indicator Virginia Crab and epinasty and decline in Spy 227, but is a latent virus and remains symptomless in most commercial cultivars. Since biological indexing is a lengthy process, various attempts have been made to produce an antiserum against ASPV CP (Komorowska and Malinowski, 2009) for use in ELISA. An ELISA for the detection of ASPV is currently commercially produced by companies such as Bioreba (Switzerland). However, more recently, research has also been conducted to show that RT-PCR is a more sensitive and reliable method for the detection of ASPV (Malinowski *et al*., 1998; Menzel et *al.*, 2002; Marinho *et al*., 2003; Menzel et *al.*, 2003; Komorowska *et al*., 2010).

Neither ELISA nor RT-PCR testing is currently used for ASPV testing in South Africa. The aim of this study was therefore to assess the levels of ASPV infection in pome fruit trees in South Africa using the highly sensitive RT-PCR. Furthermore, pear trees producing fruit with pear stony pit symptoms were assessed for ASPV infection. In order to assess the genetic variation of ASPV isolates found in South Africa, the CP gene regions of ASPV infected samples were amplified, sequenced and phylogenetically analyzed.

5.2. Materials and methods

5.2.1. Sampling

Samples used in this study included pome fruit samples from nucleus greenhouse material, foundation block material, mother block material as well as diagnostic samples submitted to the SAPO Trust laboratory for RT-PCR virus screening. Sampling was done over three of SAPO Trust laboratory's active testing seasons between September 2011 and December 2014. A total of 77 samples from three categories were selected and screened for the presence of ASPV:

1. ASGV infected samples - a total of 65 pome fruit leaf samples that tested positive for ASGV with RT-PCR were used to determine whether they were additionally infected with ASPV;
2. ASPV infected samples - a total of 11 pome fruit leaf samples submitted to the SAPO Trust laboratory for ASPV testing using RT-PCR;
3. Pear trees showing visual symptoms associated with ASPV observed during orchard inspections - a total of 4 pear tree leaf samples of which the fruit showed visual symptoms of pear stony pit and fruit deformation were included for ASPV testing using RT-PCR.

5.2.2. Sample preparation

Samples were prepared by stacking ten leaves onto each other and by using a sterile scalpel, a representative sample (leaf tissue from each leaf in the sample) of 0.1 g was cut. Total RNA was extracted from each sample using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was eluted in a total volume of 40 µl.

5.2.3. Primers

For the identification of infections of ASGV and ASPV the primers described by Menzel *et al.* (2002) were used in a multiplex RT-PCR. The multiplex sequences of the RT-PCR primer pairs are shown in Table 5.1. For sequencing of the ASPV CP gene of infected samples, cDNA was obtained by reverse transcription using the ASPV reverse transcriptase primer described by Gadiou *et al*. (2010). Sequences were obtained from cDNA using the ASPV CP gene sequencing primer pair also described by Gadiou *et al*. (2010). Both the ASPV reverse transcriptase primer and the ASPV sequencing primers are shown in Table 5.1.

Table 5.1. Primers used for ASGV and ASPV Multiplex RT-PCR and the amplification of ASPV segments

Primer	**Primer sequence in 5'-3' orientation**	**Primer position**	**Product size**	**Reference**
Primers for ASGV and ASPV Multiplex RT-PCR				
ASGV Forward	GCCACTTCTAGGCAGAACTCTTTGAA	6039-6064	273 bp	Menzel *et al*., 2002
ASGV Reverse	AACCCCTTTTTGTCCTTCAGTACGAA	6286-6311		
ASPV Forward	ATGTCTGGAACCTCATGCTGCAA	8869-8895	370 bp	Menzel *et al*., 2002
ASPV Reverse	TTGGGATCAACTTTACTAAAAAGCATAA	9211-9238		

Primer	Primer sequence in 5'-3' orientation	Primer position	Product size	Reference
Primers for Apple stem pitting virus CP gene sequencing PCR				
ASPV Reverse Transcriptase	GTCCCGGTTAGGTTGGGATC	-	n.a.	Gadiou *et al*., 2010
ASPV Forward	CWAAYCCWTTTGAAACTGG	8312-8330	840 bp	
ASPV Reverse	GCTTGGGTCCAAYYTTTC	9134-9151		

5.2.4. Multiplex RT PCR for the detection of ASGV and ASPV

A Multiplex RT-PCR mixture was prepared containing 2.5 µl 10x PCR buffer (Bioline, UK), 1.25 µl of 0.1 M DTT, 1 µl of 25 mM $MgCl_2$, 1 µl ASPV forward primer (20µM) , 1 µl ASPV reverse primer (20 µM), 1 µl ASGV forward primer (20 µM), 1 µl ASGV reverse primer (20 µM), 1 µl of 5 mM dNTP's (Bioline, UK), 0.25 µl of 5 U/µl Taq™ DNA polymerase (Bioline, UK), 0.125 µl of 200 U/µl SuperScriptTM III reverse transcriptase (Invitrogen, USA) and 12.875 µl Milli-Q® water. A total of 2 µl of isolated RNA solution was added to 23 µl of the RT-PCR reaction mixtures. The one-step multiplex RT-PCR was performed by reverse transcriptase at 48°C for 30 minutes followed by 40 cycles of the following parameters: denaturation at 96°C for 30 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 60 seconds with a final elongation step at 72°C for 10 minutes. The amplifications were performed using a Applied Biosystems Veriti Thermocycler (Thermo Fisher Scientific, US). The expected amplicon sizes of the ASGV and ASPV amplicons are listed in Table 5.1.

5.2.5. Agarose gel electrophoresis

A 1% (w/v) agarose gel was prepared with 1 × TAE buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and Milli-Q® water. Ethidium bromide (Sigma) was added to visualize products under UV light. The electrophoresis buffer consisted of 1 × TAE buffer. Ten microliters of the PCR reaction mixture was mixed with loading buffer (Bioline, UK) and loaded on the agarose gel. Electrophoresis was performed at 100 V for 80-100 minutes using a power source (Cleaver Scientific, UK). A 100 bp DNA size marker ladder (Bioline, UK) was used to determine the size of amplified DNA fragments. The DNA bands were visualized under a UV transilluminator and portable darkroom (Cleaver Scientific, UK). Amplification was regarded as successful where visual bands of the anticipated fragment length were observed.

5.2.6. RT-PCR for the amplification of the ASPV CP gene of isolates

A RT-PCR mixture was prepared containing 2.5 µl 10x PCR buffer (Bioline, UK), 1.25 µl of 0.1 M DTT, 1 µl of 25 mM $MgCl_2$, 1 µl ASPV RT primer (20 µM), 1 µl ASPV forward primer

(20 µM), 1 µl ASPV reverse primer (20 µM), 1 µl of 5 mM dNTP's (Bioline, UK), 0.25 µl of 5 U/µl Taq™ DNA polymerase (Bioline, UK), 0.125 µl of 200 U/µl SuperScriptTM III reverse transcriptase (Invitrogen, USA) and 13.875 µl Milli-Q® water. A total of 2 µl of isolated RNA solution was added to 23 µl of the RT-PCR reaction mixtures. The one-step multiplex RT-PCR was performed by reverse transcriptase at 48°C for 30 minutes followed by 40 cycles of the following parameters: denaturation at 96°C for 30 seconds, annealing at 56°C for 45 seconds and extention at 72°C for 60 seconds, with a final elongation step at 72°C for 10 minutes. Amplifications were performed using a Applied Biosystems Veriti Thermocycler (Thermo Fisher Scientific, US). The size of the CP amplicon of ASPV is listed in Table 5.1.

5.2.7. RT-PCR product purification

Prior to sequencing the RT-PCR amplicons of the CP genes they were purified using a Wizard SV Gel and PCR Clean-up System (Promega, UK) according to the manufacturer's instructions. The final purified PCR products were eluted in 40 µl of Milli-Q® water. Purified products were separated on a 1% (w/v) agarose gel (prepared as above) containing 1 µg/ml ethidium bromide and 2 µl of the eluted DNA and visualized as described above to assess DNA yield. The DNA products were stored in nuclease-free Eppendorf tubes at -20°C until sequenced.

5.2.8. Dye terminator sequencing reaction used for sequencing

The sequencing reaction mixture consisted of 5 µl of 5 × sequence dilution buffer (Applied Biosystems, California, US), 2 µl Terminator Dye (Big Dye® Terminator v3 Cycle Sequencing kit, Applied Biosystems, California, US), 1 µl of the cDNA template solution and 1 µl primer (0.8 µM) and 1 µl of MilliQ® water. The same combinations of forward and reverse primers used for amplification were also used for sequencing reactions (Table 3.4). The thermal cycling program consisted of 35 cycles of 96°C for 10 seconds, 52°C for 30 seconds, 60°C for 4 minutes followed by an elongation step at 60°C for 7 minutes. Cycle sequencing products were analyzed in an ABI 3730 DNA Analyser (Applied Biosystems, California, US) at the Central Analytical Facility, University of Stellenbosch.

5.2.9. CP gene sequence analysis and alignment

The sequence chromatograms resulting from the forward and reverse dye terminator sequencing reactions were edited using ChromasPro version 1.5 (Technelysium Pty., Ltd.) and combined to produce consensus sequences. The generated sequences were verified as ASPV CP genes using BLASTn searches on GenBank (http://blast.ncbi.nlm.gov/Blast.cgi). The generated sequences were aligned with CP gene sequences obtained from GenBank (Table 5.2) using BioEdit version 7.0.5.2 software package (Hall, 1999). CP gene sequences

of *Peach asteroid spot virus* (PASV) (AF318062) and ALV (AF057035) were used as outgroups for ASPV phylogenetic analyses (Table 5.2). Alignment of the outgroups and ASPV CP gene sequences was performed using the Clustal W version 1.4 alignment function of the BioEdit package. The CP gene alignment was refined manually in triplets starting at the first position of the codon. This was done in order to translate the alignment to an amino acid alignment for use in the analysis.

Table 5.2. Details of the CP gene sequences of ASPV isolates obtained from GenBank used for phylogenetic analysis.

GenBank Accession number	**Virus**	**Host**	**Country of origin**	**Reference**
AF318061	PASV	Peach	France	Gentit *et al*., 2001
AF057035	ALV	Peach	Unknown	Nemchinov *et al*., 2000
EU095327	ASPV	China	Pear	Zhao and Niu (direct submission)
JF946775	ASPV	China	Pear	Liu *et al*., 2012
JF946772	ASPV	China	Pear	Liu *et al*., 2012
AB045371	ASPV	Unknown	Apple	Yoshikawa *et al*. (unpublished)
FR694186	ASPV	India	Apple	Dhir *et al*. (unpublished)
KF915809	ASPV	China	Apple	Chen *et al*. (unpublished)
EU708018	ASPV	Pear	China	Li (unpublished)
AJ968944	ASPV	Pear and Apple	Czech Republic	Hassan and Salava (unpublished)
JX673828	ASPV	Pear	China	Ma *et al*. (unpublished)
FJ619188	ASPV	Apple	China	Li *et al*. (unpublished)
HM125154	ASPV	Apple	China	Li and Dong (unpublished)
AF345894	ASPV	Pear	Unknown	Komorowska and Malinowski (unpublished)
HM125159	ASPV	Apple	China	Li and Dong (unpublished)
JX673809	ASPV	Pear	China	Ma *et al*. (unpublished)
FJ970956	ASPV	Apple	India	Gadiou *et al*., 2010
FJ970957	ASPV	Apple	Poland	Gadiou *et al*., 2010
FJ970949	ASPV	Apple	Poland	Gadiou *et al*., 2010
FJ970953	ASPV	Apple	België	Gadiou *et al*., 2010
FJ970955	ASPV	Apple	België	Gadiou *et al*., 2010
FJ970950	ASPV	Apple	Ukraine	Gadiou *et al*., 2010
FJ970951	ASPV	Apple	Ukraine	Gadiou *et al*., 2010
FJ970952	ASPV	Apple	Ukraine	Gadiou *et al*., 2010
FJ970960	ASPV	Apple	Turkey	Gadiou *et al*., 2010
FJ970961	ASPV	Apple	Turkey	Gadiou *et al*., 2010

5.2.10. Phylogenetic analysis using parsimony

Phylogenetic analyses using parsimony was conducted with PAUP version 4.0b10 (Swofford, 2002). The CP gene sequences of 24 isolates obtained from GenBank (Table 5.2) and 24 South African isolates were used in a phylogenetic analysis using parsimony. A heuristic search was performed to search for the shortest trees from the sequence alignment matrix. The search criteria included the use of the 1000 additional sequence replicates using TBR branch swapping algorithm. Gaps were treated as missing data. All characters were unordered and equally weighted. Bootstrap support values were calculated from the 1000 heuristic search replicates and 10 random taxon additions to determine clade support. Bootstrap values less than 50% were considered weakly supported and were not indicated on phylograms. Values from 50% to 74% were considered moderately supported. Branches with bootstrap values of 75% and higher were considered as well supported. The parsimony analyses were used to calculate CI and RI values (Farris, 1994). The second parsimony analysis was performed on the CP amino acid alignment. In a third parsimony analysis the third codon position of the CP gene sequence alignment was excluded. CP gene sequences of PASV (AF318062) and ALV (AF057035) were used as outgroups for all three parsimony analyses.

5.2.11. Phylogenetic analysis using maximum likelihood

Phylogenetic analysis using maximum likelihood was performed on the alignment matrices using RAxML version 8.0.24, a tool for phylogenetic analysis and post-analysis of large phylogenies (Stamakis, 2014) on the CIPRES Science Gateway (Miller *et al*., 2010). Trees retrieved from RAxML were drawn in PDF format using Tred tree viewing (http://www.reelab.net/tred).

A total of three analyses were performed. In the first analysis the CP gene sequence matrix was analyzed. The second analysis was performed on the same data matrix translated to an amino acid alignment. In the third analysis the nucleotide positions were partitioned into first, second and third positions of each codon.

5.3. Results

5.3.1 Detection of ASGV and ASPV infection using RT-PCR

The presence of bands of the expected sizes of ASGV and ASPV RT-PCR amplicons of 273 bp and 370 bp respectively on agarose gels after electrophoresis indicated that samples were infected with one or both viruses. Double infection of ASGV and ASPV was found in 19 of the 77 samples. The multiplex RT-PCR results of seven samples which were found to be

infected with ASGV and/or ASPV are shown in Figure 5.1. The samples in which ASGV and ASPV was detected are shown in Table 5.3. Pear samples MC68, MC69, MC70 and MC71 which showed severe symptoms of pear stony pit and fruit deformation as shown in Figure 5.2 and were found to be infected with ASPV by RT-PCR are indicated in blue (Table 5.3).

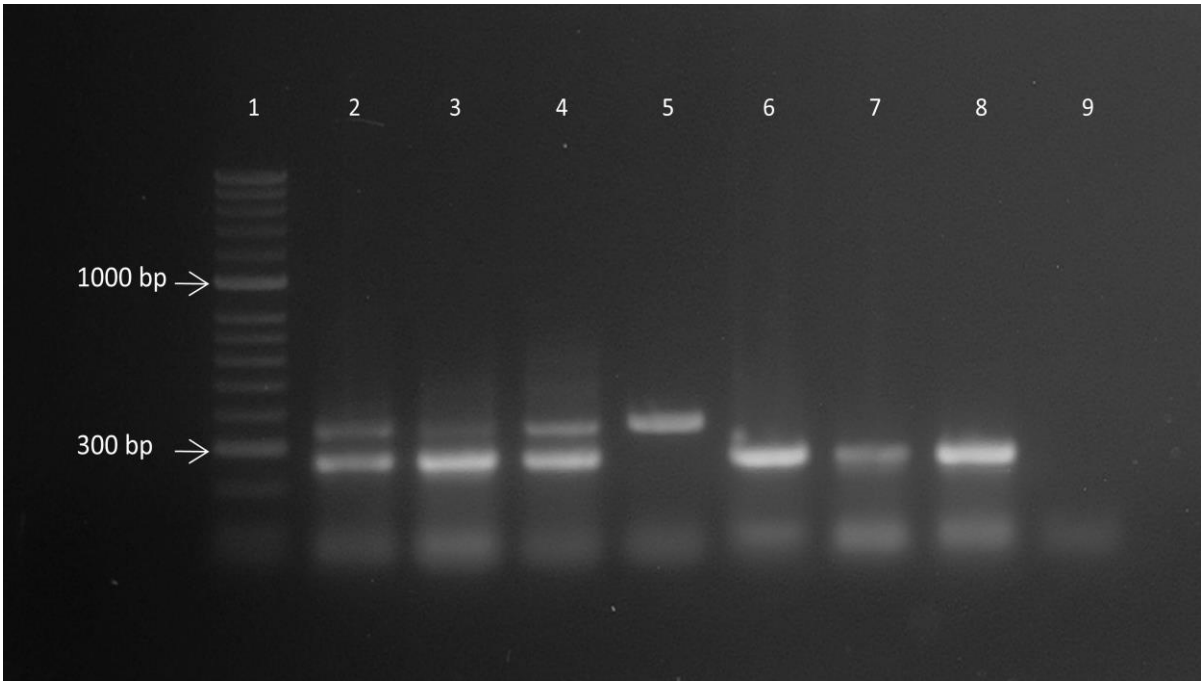

Figure 5.1. Gel electrophoresis of samples after ASGV and ASPV multiplex RT-PCR to determine double-infection of ASGV infected isolates with ASPV. ASGV infected samples gave RT-PCR products at 273 bp while ASPV infected samples gave RT-PCR products at 370 bp. DNA markers are shown at 300 bp and 1000 bp. Lane 1: Bioline 100 bp Hyperladder^{TM} II. Lane 2: Sample MC26. Lane 3: Sample MC35. Lane 4: Sample MC61. Lane 5: Sample MC67. Lane 6: Sample MC16. Lane 7: Sample MC41. Lane 8: Sample MC17. Lane 9: Negative control.

Table 5.3. The list of pome fruit samples used in this study. Samples in which virus detection and/or sequence analysis was successful are marked with ■. Samples in which virus detection and/or sequence analysis was unsuccessful are marked with □. Pear samples that showed pear stony pit and fruit deformation symptoms are indicated in blue.

			ASGV	ASPV	ASPV	
Collection number	**Fruit type**	**Sample origin**	**Multiplex RT-PCR**	**Multiplex RT-PCR**	**CP gene RT-PCR**	**CP gene Sequencing PCR**
MC1	Apple	Stellenbosch	■	■	■	■
MC2	Apple	Stellenbosch	■	□	□	□
MC3	Apple	Stellenbosch	■	□	□	□
MC5	Apple	Stellenbosch	■	□	□	□
MC7	Apple	Stellenbosch	■	□	□	□
MC8	Apple	Stellenbosch	■	□	□	□
MC9	Apple	Stellenbosch	■	□	□	□
MC10	Apple	Stellenbosch	■	□	□	□
MC11	Apple	Stellenbosch	■	□	□	□
MC12	Apple	Stellenbosch	■	□	□	□
MC13	Apple	Ceres	■	□	□	□
MC14	Apple	Ceres	■	■	■	□
MC15	Apple	Villiersdorp	■	□	□	□
MC16	Apple	Villiersdorp	■	□	□	□
MC17	Apple	Ceres	■	□	□	□
MC18	Apple	Grabouw	■	□	□	□
MC19	Apple	Grabouw	■	■	■	□
MC20	Apple	Unknown	■	□	□	□
MC21	Apple	Unknown	■	□	□	□
MC22	Apple	Unknown	■	□	□	□
MC23	Apple	Unknown	■	□	□	□
MC24	Apple	Unknown	■	□	□	□
MC25	Apple	Stellenbosch	■	□	□	□
MC26	Apple	Stellenbosch	■	■	■	■
MC27	Apple	Stellenbosch	■	□	□	□
MC28	Apple	Stellenbosch	■	■	■	■
MC29	Apple	Stellenbosch	■	■	■	■
MC30	Apple	Unknown	■	□	□	□
MC31	Apple	Unknown	■	□	□	□
MC32	Apple	Unknown	■	□	□	□
MC33	Apple	Unknown	■	□	□	□
MC34	Apple	Unknown	■	□	□	□
MC35	Apple	Unknown	■	■	■	■
MC36	Apple	Stellenbosch	■	□	□	□
MC37	Apple	Stellenbosch	■	□	□	□
MC38	Apple	Stellenbosch	■	□	□	□
MC39	Apple	Stellenbosch	■	■	■	■
MC40	Apple	Stellenbosch	■	■	■	■
MC41	Apple	Unknown	■	□	□	□

Collection number	Fruit type	Sample origin	ASGV	ASPV	ASPV	
			Multiplex RT-PCR	Multiplex RT-PCR	CP gene RT-PCR	CP gene Sequencing PCR
MC43	Apple	Unknown	■	□	□	□
MC44	Apple	Stellenbosch	■	□	□	□
MC45	Apple	Unknown	■	■	■	■
MC46	Apple	Unknown	■	■	■	□
MC47	Apple	Stellenbosch	■	■	■	■
MC48.1	Apple	Stellenbosch	■	■	■	■
MC48.2	Apple	Stellenbosch	■	□	□	□
MC49.1	Apple	Stellenbosch	■	■	■	■
MC49.2	Apple	Stellenbosch	■	■	■	■
MC50	Apple	Stellenbosch	■	■	■	■
MC51	Apple	Stellenbosch	■	□	□	□
MC52	Apple	Stellenbosch	■	□	□	□
MC53	Apple	Stellenbosch	■	□	□	□
MC54	Apple	Stellenbosch	■	□	□	□
MC55	Apple	Stellenbosch	■	□	□	□
MC56	Apple	Stellenbosch	■	□	□	□
MC57	Apple	Stellenbosch	■	■	■	□
MC58	Apple	Stellenbosch	■	■	■	■
MC59	Apple	Grabouw	■	□	□	□
MC60	Apple	Grabouw	■	□	□	□
MC61	Apple	Ceres	■	■	■	□
MC62	Apple	Grabouw	■	□	□	□
MC63	Apple	Grabouw	■	□	□	□
MC64	Apple	Grabouw	■	□	□	□
MC65	Apple	Riviersonderend	■	□	□	□
MC66	Apple	Riviersonderend	■	□	□	□
MC67	Apple	Stellenbosch	□	■	■	■
MC68*	Pear	Grabouw	□	■	■	■
MC69*	Pear	Grabouw	□	■	■	■
MC70*	Pear	Grabouw	□	■	■	■
MC71*	Pear	Grabouw	□	■	■	□
MC72	Pear	Ceres	□	■	■	□
MC73	Pear	Ceres	□	■	■	■
MC74	Pear	Stellenbosch	□	■	■	■
MC75	Pear	Riviersonderend	□	■	■	■
MC76	Pear	Villiersdorp	□	■	■	■
MC77	Pear	Villiersdorp	□	■	■	■
MC78	Pear	Villiersdorp	□	■	■	■

*Pear samples from Grabouw, Western Cape, that showed pear stony pit symptoms during visual inspection.

Figure 5.2. Pears showing pear stony pit- and fruit deformation symptoms from Grabouw, Western Cape, South Africa (Samples MC68, MC69, MC70 and MC71).

5.3.2 Sequence analysis of ASPV amplified products

The RT-PCR amplicons of the ASPV CP genes separated by gel electrophoresis showed bands of the expected size of 870 bp (Figure 5.3). The CP genes of 14 of the 20 samples that tested positive for the presence of ASPV were successfully sequenced (Table 5.3).

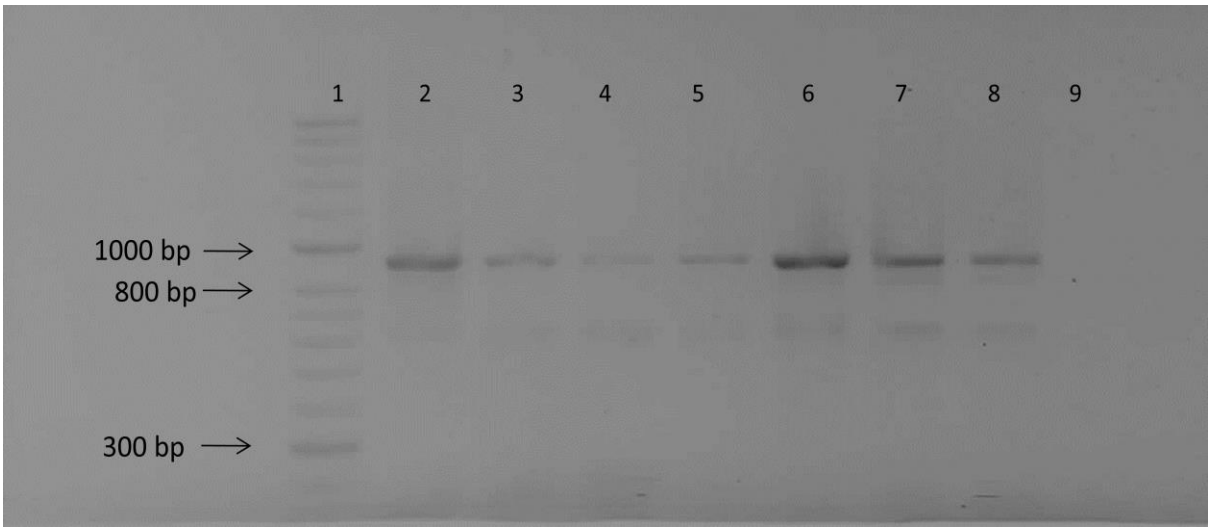

Figure 5.3. Gel electrophoresis of the ASPV CP gene RT-PCR products of seven ASPV infected samples. ASPV infected samples gave RT-PCR products at 870 bp. Lane 1: Bioline 100 bp Hyperladder™ II DNA markers. Lane 2: Sample 1. Lane 3: Sample MC26. Lane 4: Sample MC29. Lane 5: Sample MC35. Lane 67: Sample MC40. Lane 7: Sample MC68. Lane 8: Sample MC70. Lane 9: Negative control.

5.3.3. Phylogenetic analysis of ASPV CP gene sequences using parsimony

In the phylogenetic analysis of ASPV CP gene alignment using parsimony, the heuristic search retrieved only one tree with a tree length of 3407. The analysis revealed that a total of 320 (36.66%) characters were constant, 100 (11.45%) characters were parsimony uninformative and 453 (51.89%) characters were parsimony informative. The single tree is shown in Figure 5.4. The tree statistics revealed a CI of 0.289 and a RI of 0.554.

Phylogenetic analyses performed on ASPV CP amino acid sequence data using parsimony, the heuristic search retrieved a total of 1063 trees with a tree length of 764. The analysis revealed that a total of 115 (39.52%) characters were constant, 69 (23.71%) characters were parsimony uninformative and 107 (36.77%) characters were parsimony informative. One of the most parsimonious trees is shown in Figure 5.5. The tree statistics revealed a CI of 0.559 and a RI of 0.621.

An ASPV CP gene sequences alignment in which the third position in the codon was excluded was used in a phylogenetic analysis using parsimony. The heuristic search retrieved a total of four trees with a tree length of 965. The analysis revealed that a total of 304 (52.32%) characters were constant, 183 (31.50%) characters were parsimony uninformative and 94 (16.18%) characters were parsimony informative. One of the most parsimonious trees is shown in Figure 5.6. The tree statistics revealed a CI of 0.424 and a RI of 0.589.

The trees shown in Figure 5.3, 5.4 and 5.5 all show that the isolates grouped into two monophyletic groups. South African isolates grouped into the larger monophyletic group. Clades from this group lacked resolution as few relationships between clades were supported. The phylogeny did however retrieve some strongly supported clades and sub-clades. In Figure 5.3, a total of two clades showed bootstrap support >75% and a total of 16 clades and sub-clades showed bootstrap support values >75%. In Figure 5.4, a total of two clades showed bootstrap support >75% and a total of six clades and sub-clades showed bootstrap support values >75. In Figure 5.5, none of the clades showed bootstrap support >75% and ten clades and sub-clades showed bootstrap support values >75.

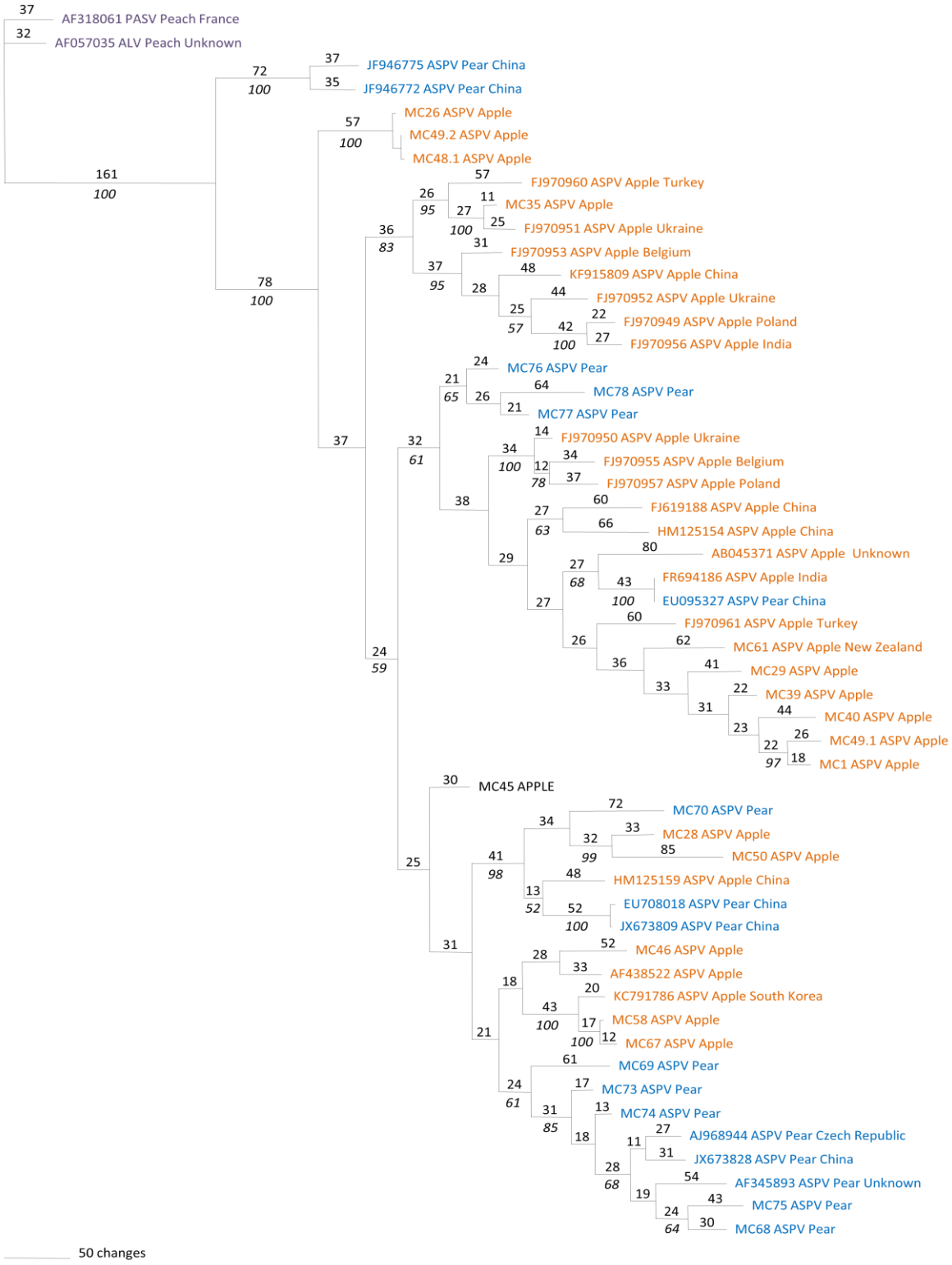

Figure 5.4. The single shortest tree retrieved from a parsimony analysis performed on ASPV CP gene sequence data matrix. Branch lengths ≥10 are indicated above branches and bootstrap values below branches in italics. South African isolates are indicated with the lettering starting with MC. Clades with bootstrap support of <50% are not indicated in the tree.

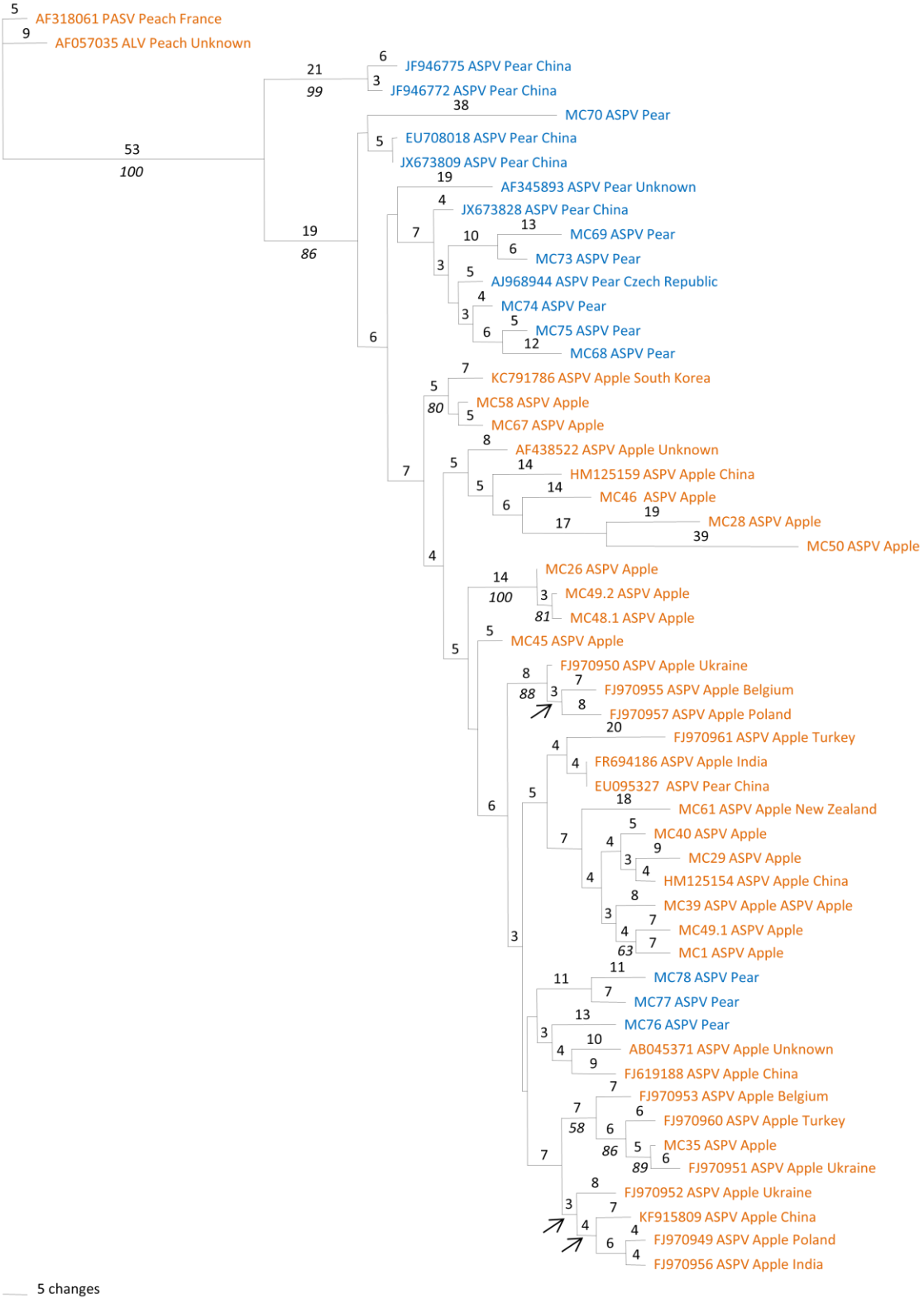

Figure 5.5. One of the shortest trees retrieved from a heuristic search performed on ASPV CP amino acid sequence data. Branch lengths ≥3 are indicated above branches and bootstrap values below branches in italics. Branches that collapsed in the strict consensus are indicated with arrows. South African isolates are indicated with the lettering starting with MC. Clades with bootstrap support of <50% are not indicated in the tree.

Figure 5.6. One of the shortest trees retrieved from the parsimony analysis performed on ASPV CP gene sequence data matrix of which the third position in the codon was excluded. Branch lengths ≥5 are indicated above branches and bootstrap values below branches in italics. South African isolates are indicated with the lettering starting with MC. Clades with bootstrap support of <50% are not indicated in the tree.

5.3.4. Phylogenetic analysis of ASPV CP gene using maximum likelihood

The tree with the highest likelihood was retrieved from the maximum likelihood analysis of ASPV CP gene sequences. All trees retrieved from the maximum likelihood analyses of ASPV CP gene sequences showed two monophyletic groups. The tree retrieved from the ASPV CP gene sequences matrix analysis (codon unpartitioned) is shown in Figure 5.7, the tree retrieved from the ASPV CP gene sequence matrix analysis translated to amino acids is shown in Figure 5.8 and the tree retrieved from analyzing the different codon positions of ASPV CP gene sequences matrix (Figure 5.9).

In Figure 5.7, a total of two clades showed bootstrap support >75% and 18 clades and sub-clades showed bootstrap support values >75%. In Figure 5.8, two clades showed bootstrap support >75% and 19 clades and sub-clades showed bootstrap support values >75%. In Figure 5.9, three clades showed bootstrap support >75% and 20 clades and sub-clades showed bootstrap support values >75%. In Figure 5.9, the first monophyletic group consists of a clade of two ASPV sequences from China. The second monophyletic group consists of all South Africa isolates sub-grouped into five clades with bootstrap support between 65% (clade 5) and 100% (clade 3).

Clade 1 consists of apple as well as pear isolates and is well supported. The South African pear isolates MC76, MC77 and MC78 form a highly supported sub-clade within clade 1. The CP gene sequences of pear isolate EU095327 from China and apple isolate FR694186 from India comprising clade 1 is highly supported. Clade 2 is strongly supported and contains a sub-clade with 100% bootstrap support consisting of the apple CP gene sequences of the South African isolate MC 35, isolate FJ970951 from Ukraine and isolate FJ970960 from Turkey. Clade 4 only consists of the CP gene sequences of South African apple isolates MC26, MC 48.1 and MC49.2 and is highly supported. The CP gene sequence of apple isolate MC 45 (Clade 3) did not show any support and is therefore seen as a separate isolate with unique characteristics compared to the other CP gene sequence data included in the analysis.

Clade 5 consists of CP gene sequences of both apple and pear isolates, is weakly supported and consists of two sub-clades. The first sub-clade is strongly supported and consists of CP gene sequences of South African apple isolates MC28 and MC50, pear isolate MC70 and apple and pear isolates from China (HM125159, EU708018 and JX673809). The second sub-clade is moderately supported and consists CP gene sequences of South African pear isolates MC68, MC69, MC73, MC74 and MC75 and pear isolates from China and the Czech Republic.

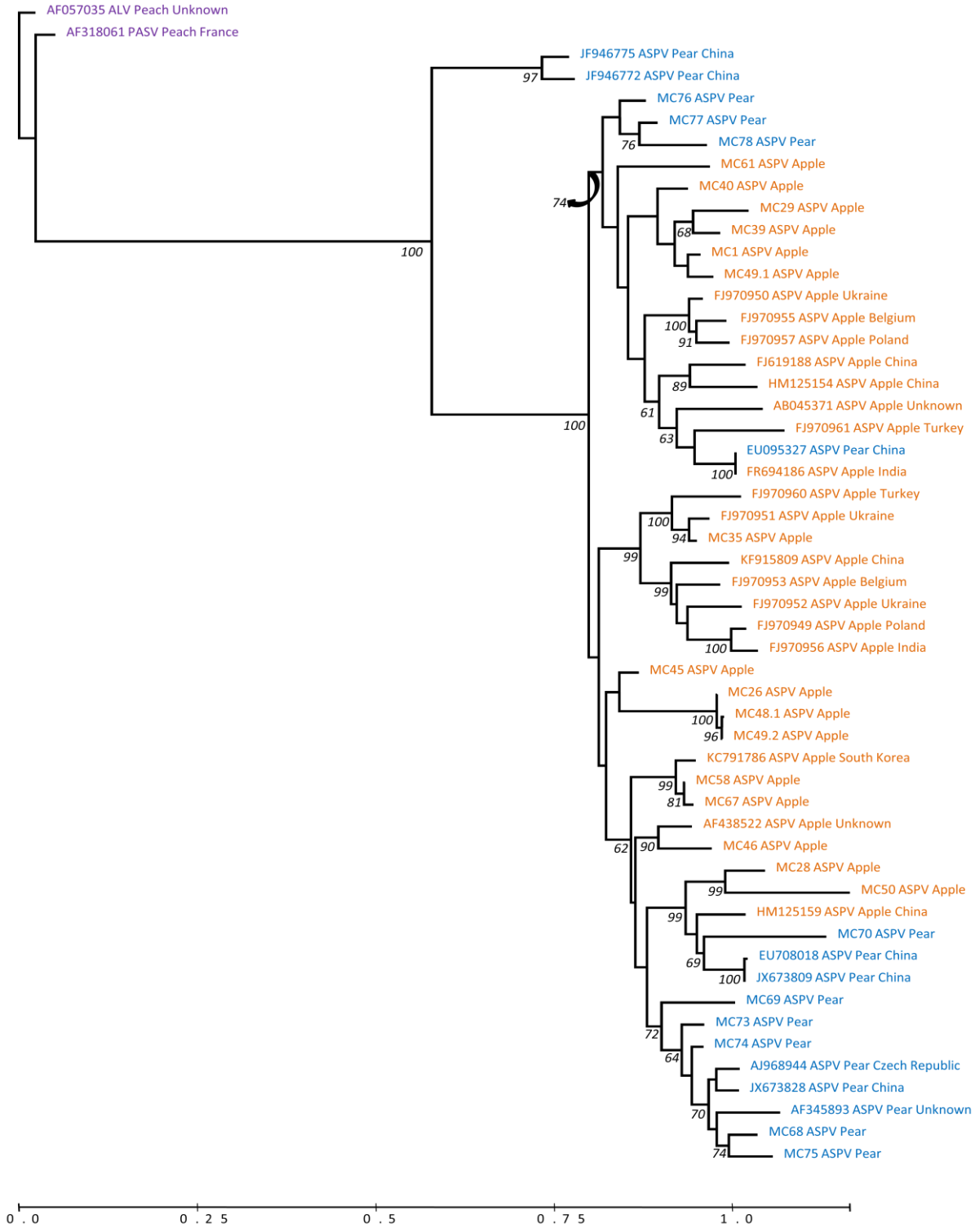

Figure 5.7. The most likely tree retrieved from a search performed on the ASPV CP gene sequence data matrix (codon partitioned) using maximum likelihood. Bootstrap values are indicated below branches in italics. South African isolates are indicated with the lettering starting with MC. Apple and pear samples are indicated in orange and blue respectively. Clades with bootstrap support of <50% are not indicated in the tree.

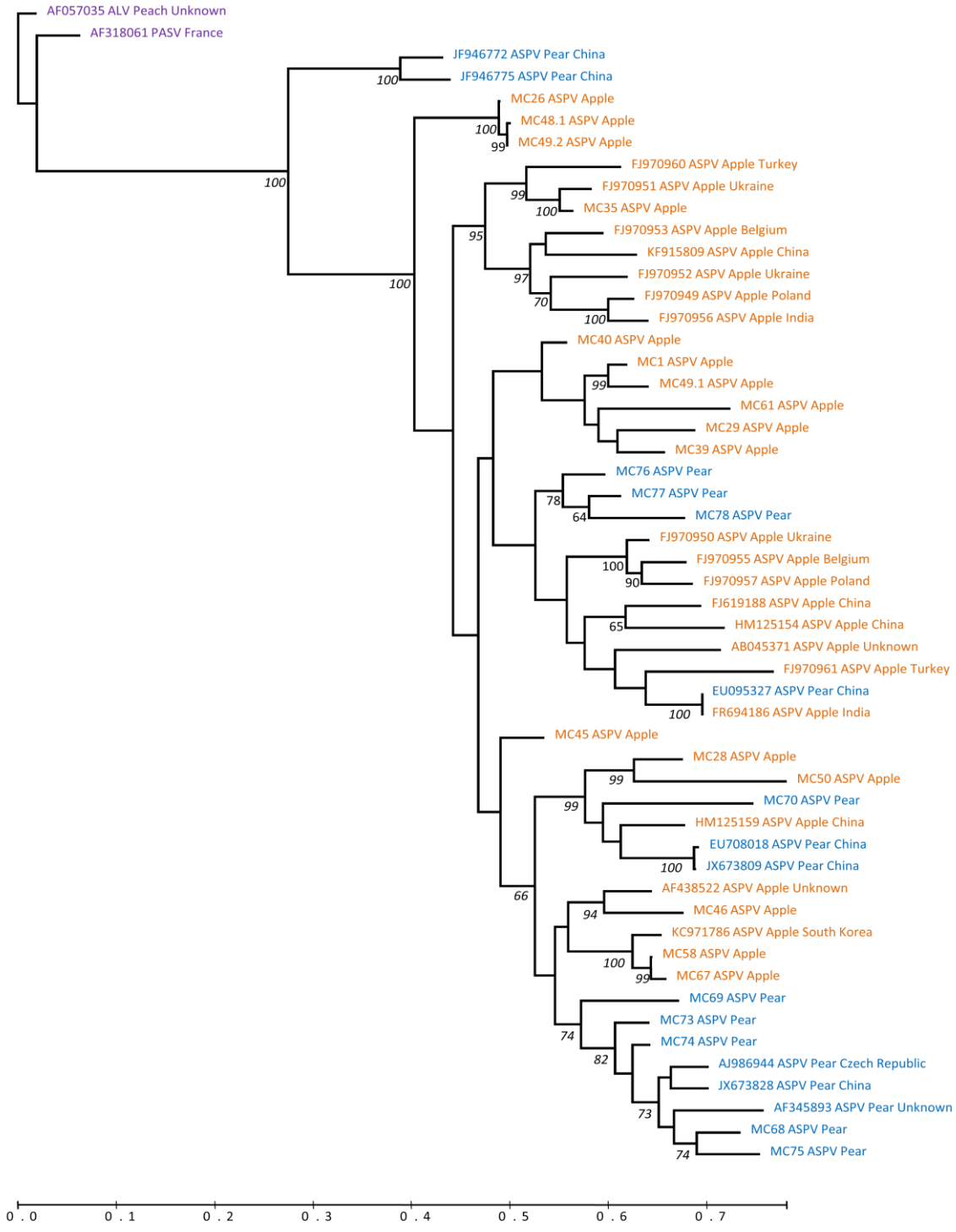

Figure 5.8. The most likely tree retrieved from a search performed on the ASPV CP amino acid sequences using maximum likelihood. Bootstrap values are indicated below branches in italics. South African isolates are indicated with the lettering starting with MC. Apple and pear samples are indicated in orange and blue respectively. Clades with bootstrap support of <50% are not indicated in the tree.

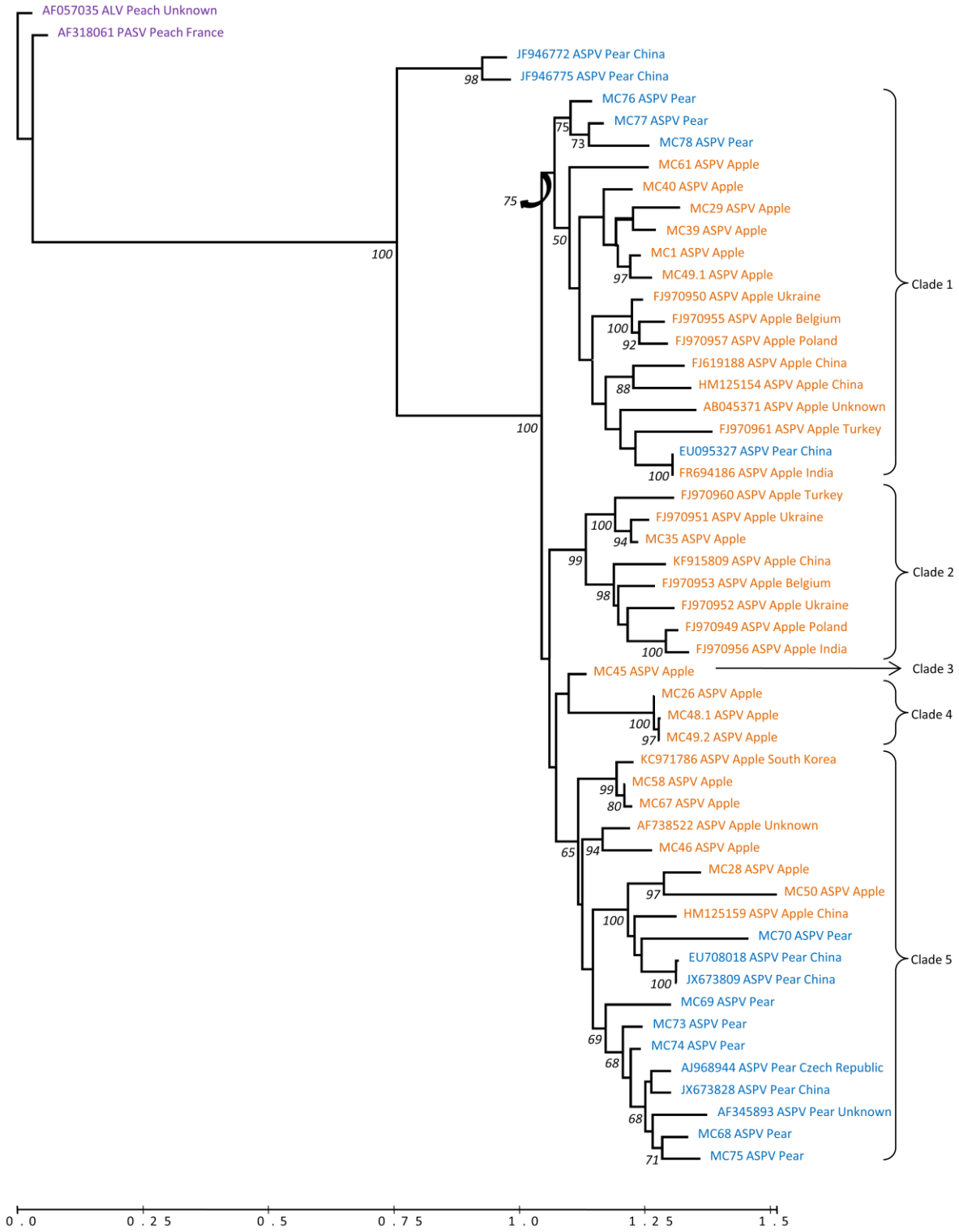

Figure 5.9. The most likely tree retrieved from a search performed on the ASPV CP gene sequence data matrix (codon partitioned) using maximum likelihood. Bootstrap values are indicated below branches in italics. South African isolates are indicated with the lettering starting with MC. Apple and pear samples are indicated in orange and blue respectively. Clades with bootstrap support of <50% are not indicated in the tree.

5.4. Discussion

This study is the first in which ASPV has been detected by RT-PCR in South Africa. Double infections with ASGV and ASPV were recorded in 24.7% of samples which were all from apple trees. This is in agreement with other studies in which ASPV infection often occurs with ASGV. RT-PCR results from studies performed in the Czech Republic and Turkey showed mixed infection with ASGV and ASPV in 14.7% and 36.8% of the samples respectively (Kundu, 2003; Caglayan *et al.,* 2006). However, the 24.7% determined in this study may be too high as an extensive survey for ASGV was not performed and single infection with ASGV may therefore occur more often. Pear samples used in this study were found to be infected with ASPV only. A study performed by Hassan *et al*. (2008) tested apple and pear samples for the presence of the pome fruit viruses ACLSV, ApMV, ASGV and ASPV. Only ASPV and ACLSV could be detected in pear samples.

Although a number of apple and pear disease syndromes are suspected to be caused by ASPV (Jelkman, 1994; Leone *et al*., 1995; Leone *et al*., 1998; Paunovic and Rankovic., 1998; Paunovic *et al*., 1999; Desvignes *et al*., 1999), it is only Pear vein yellows that has been demonstrated to be caused by ASPV (Leone *et al*., 1998; Wu *et al*., 2010). A total of four pear samples (MC68, MC69, MC70 and MC71) used in this study showed symptoms of pear stony pit and fruit deformities. Paunovic *et al*. (1999) identified a sample of the pear cultivar Württemberg associated with pear stony pit to be infected with ASPV. However, the causal agent of pear stony pit disease has not been established, neither in South African nor elsewhere in the world. Further studies, including more samples from a wider geographical area as well as studies done on the possible comparison between different virus particles found in ASPV and pear stony pit infected plants using electron microscopy, will have to be performed to confirm whether ASPV is responsible for this disease pathology. Conclusive proof that ASPV is the causal agent of pear stony pit disease would be to isolate viral particles from infected fruit and infection of uninfected trees with these particles followed by pear stony pit symptom expression on their fruit according to Koch's postulates. The parsimony analysis of ASPV CP gene sequences retrieved a tree that was neither well resolved nor supported. The phylogenetic analysis revealed that the CI and RI values were relatively low indicating that there was considerable homoplasy in the CP gene sequence data. In an effort to improve this, the phylogenetic analysis was refined. In an attempt to address the homoplasy that was evident from the first analysis, the nucleic acid sequence data were converted to amino acid sequence data and analyzed phylogenetically. Additionally an analysis was run in which the third position of the codon was excluded from the alignment and only the first and second coding positions were used. Both of these approaches decreased homoplasy in the data set as is evident from the higher CI and RI

values, but due to the reduction in the number of phylogenetically informative characters this reduced resolution to some extent. However, resolution did improve from the first to the third parsimony analysis as was evident from the increased number of clades and sub-clades with >75% bootstrap support.

In an attempt to improve the resolution of the phylogeny, the phylogenetic analysis was repeated using maximum likelihood analysis. In the first analysis of CP gene sequences (codon unpartitioned) the most likely tree retrieved showed South African ASPV sequences to group into five clades which were not all well supported. As with the parsimony analysis, the second maximum likelihood analysis was performed on the CP gene data translated to amino acid sequences. The most likely tree retrieved showed increased clade support. The ASPV CP gene data was further analyzed by partitioning of the first, second and third position of the codon. This approach was successful in improving resolution as the tree with the highest resolution was retrieved in this analysis.

These results show that the third position in the codon of the CP gene sequence data increases homoplasy in the data set. Refinement of the data by excluding the third position and/or converting sequence data to amino acid sequence data effectively reduces the influence of the third position and improves resolution. The data presented also indicates that the use of maximum likelihood of phylogenetic analysis reduces the effect of variation in the third position of the codon and therefore yields a tree with higher resolution. It appears that the model choice for the third position chosen by the maximum likelihood analyses improves tree building as is evident by better resolution and higher bootstrap support values. In the field of phylogenetic analysis, it is now commonly accepted that because parsimony only searches for shortest trees in an alignment matrix, whilst likelihood analyses first establish the model of evolution based on the alignment matrix and then derives the most likely tree based on the model, parsimony often gives a less reliable phylogeny (Whelan *et al*., 2001). Parsimony is particularly susceptible to problems in data sets which show high rates of sequence evolution (Palmer *et al*., 2004). An inspection of the nucleic acid sequence matrix reveals high levels of variation in the third position of the codon in the CP gene sequences giving further support to the conclusion that the parsimony phylogeny established from this data may not be as accurate as the maximum likelihood phylogeny. Consequently greater significance is given to the phylogenies established with maximum likelihood.

The alignment matrix of the ASPV CP gene sequences shows a very high degree of variability, particularly in the third position of the codon. High levels of genetic variability in the ASPV CP gene region were also found by a number of authors (Magome, 1997; Nemchinov *et al*., 1998; Schwarts and Jelkmann, 1998; Gadiou *et* al., 2010). RNA viruses

are known to lack an error correcting RdRp leading to a high frequency of mutations (Drake, 1993; Domingo and Holland, 1997) which is the reason for this variability. However, this variability is even higher than that found in ASGV (this study). Such high levels of variability may lead to the homoplasy observed in the ASPV CP gene sequence matrix.

The pear and apple isolates tend to cluster per fruit type with the exception of Pear isolates EU095327 from China (a sub-clade in clade 1) and pear isolates MC 70 from South Africa together with EU708018 and JX673809 from China, which grouped with apple isolates. This implies that ASPV does not possess a host specificity which limits it to either apple or pear (or quince), but rather shows broad host specificity for pome fruit. This also has practical implications in that ASPV may be transmitted from apples to pears and vice versa through contaminated pruning and grafting equipment.

This study shows that ASPV can be detected in pome fruit trees in South Africa by RT-PCR. Since South African isolates infected with ASPV show no geographical trend and are dispersed throughout the retrieved phylogenetic tree, it is most likely that they originate from different sources worldwide. ASPV and ASGV were often found as mixed infections. ASPV was for the first time detected in pear samples showing symptoms of pear stony pit and fruit deformation. The implication that this disease has for the pear industry is enormous since it makes fruit inedible and completely unmarketable. It is therefore important for the South African pome fruit industry to implement a testing program for ASPV. Although this study only presents results of ASPV infection obtained with RT-PCR, ELISA kits for ASPV are commercially available. Further studies on the sensitivity of ELISA versus RT-PCR will have to be performed to determine whether the sensitivity of the ELISA method, which is commonly accepted to be less sensitive than RT-PCT will meet the needs of the South African pome fruit industry for testing for this virus.

Chapter 6. Conclusions, future perspectives and practical implications

As was evident from this study, both ASGV and ASPV are present in South African pome fruit orchards. The results from Chapter 3 show RT-PCR to be much more sensitive than ELISA. It could be that this is also true for the detection of ASPV by DAS-ELISA versus RT-PCR. Results presented in Chapter 4 concluded that various isolates of ASGV isolates from South Africa and worldwide are closely related. It also showed that there is no geographical trend with regard to the origin of the various ASGV isolates, in South Africa as well as worldwide. Results presented in Chapter 5 presented the first evidence of ASPV being detected by RT-PCR in South Africa. Phylogenetic analysis, using parsimony and maximum likelihood respectively, showed substantial variation of the third codon position within the ASPV CP gene sequence data matrix. As was evident from previous research done worldwide, it also indicated that double infections of ASGV and ASPV using a multiplex PCR does occur in South Africa. Since the use of a multiplex PCR reduces costs by performing one single analysis instead of different analyses for each single virus, this would serve as a more sensitive and cost-effective replacement of DAS-ELISA and biological indexing within the South African Deciduous Fruit Plant Certification Scheme. Continuous improvement and optimization of this method might be useful for future use.

This study also presented the first evidence of pear samples showing pear stony pit and fruit deformation symptoms associated with ASPV. Although it is not yet known worldwide whether ASPV is the causal agent for pear stony pit disease, a total of four pear samples with pear stony pit and fruit deformation symptoms sampled in South Africa in 2014 tested positive for the presence of ASPV. Future studies should focus on the possible link between these symptoms and ASPV by applying Koch’s postulates.

Phylogenetic analyses of both ASGV and ASPV showed no geographical trend throughout the different clades since South African CP gene sequences grouped with sequences from all over the world. In view of the fact that CP gene isolates from apple and pear infected with ASPV are interspersed throughout the tree, this indicates that ASPV isolates from apple and pear are closely related. Future studies might focus on whole genome sequencing of South African ASGV and ASPV isolates in comparison to isolates from other countries of the world to obtain more information on genetic variability within these viruses.

Both ASGV and ASPV are described by and included in the South African Deciduous Fruit Plant Certification Scheme, these viruses can reduce fruit yield over several years and to cause financial losses due to the spread of the viruses through propagation material. Currently the scheme prescribes ELISA (ASGV) and biological indexing (ASGV and ASPV)

as the standard methods for testing. To reduce the spread of ASGV and ASPV within pome fruit producing regions of South Africa it is important for the whole industry to commit to plant improvement as described by the South African Deciduous Fruit Plant Certification Scheme. Furthermore the industry needs to implement strategies to eliminate the risk of initial virus infection. These include the use of certified rootstocks from reputable suppliers, buying certified fruit trees or tree propagation material from accredited suppliers or nurseries and the use of clean, sterilized pruning and pruning equipment.

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